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Review article

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Fibroblast and myofibroblast activation in normal tissue repair and fibrosis

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Abstract

The term 'fibroblast' often serves as a catch-all for a diverse array of mesenchymal cells, including perivascular cells, stromal progenitor cells and bona fide fibroblasts. Although phenotypically similar, these subpopulations are functionally distinct, maintaining tissue integrity and serving as local progenitor reservoirs. In response to tissue injury, these cells undergo a dynamic fibroblast–myofibroblast transition, marked by extracellular matrix secretion and contraction of actomyosin-based stress fibres. Importantly, whereas transient activation into myofibroblasts aids in tissue repair, persistent activation triggers pathological fibrosis. In this Review, we discuss the roles of mechanical cues, such as tissue stiffness and strain, alongside cell signalling pathways and extracellular matrix ligands in modulating myofibroblast activation and survival. We also highlight the role of epigenetic modifications and myofibroblast memory in physiological and pathological processes. Finally, we discuss potential strategies for therapeutically interfering with these factors and the associated signal transduction pathways to improve the outcome of dysregulated healing.

Sections

Introduction

Fibroblast functions and phenotypes

Myofibroblast activation

Mechanisms of (myo-) fibroblast mechanoperception

Mechanisms of myofibroblast mechanotransduction

Myofibroblast fates and memory

Conclusions and perspectives

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Introduction

The term 'fibroblast' generically describes a connective tissue cell with the ability to produce connective tissue substance (collagen). This minimal definition is attributed to Rudolph Virchow, who, in 1858, described distinct spindle-shaped cells embedded in fibrous connective tissue[1]. However, Theodor Schwann had already identified spindle-shaped cellular elements within connective tissue when he defined cells as the basic unit of the animal body in 1839 (ref. 2). In 1848, Hermann Liebert called these cellular elements "*geschwänzte fibroplastische Körperchen*", which rather cutely translates to 'tailed fibroplastic corpuscles'[3]. Ernst Ziegler then coined the term 'fibroblast' when summarizing the content of wound granulation tissue in 1885: "In most cases the hypertrophied cells are connective-tissue cells, which later produce connective tissue […], and may therefore be termed fibroblasts"[4]. Although this cell type has at least 180 years of research history since its discovery, 'fibroblasts', in fact, comprise a vastly heterogeneous group of mesenchymal cells with similar morphology but specific functions in maintaining tissue extracellular matrix (ECM) homeostasis and regulating local biological processes in the healthy adult organism. However, in conditions of tissue injury and in the presence of danger signals, such as following ECM disruption or inflammation, they suspend their specialized functions and acquire repair features aimed at rapidly containing the tissue damage by producing fibrous collagen-rich ECM and eventually contracting the newly formed ECM into mechanically stabilizing scar tissue. This phenotypic switch is called fibroblast–myofibroblast activation (in short: myofibroblast activation)[5,6].

The concept that wounds are closed by an active cell component rather than by autonomously 'condensing' wound collagen was introduced as a dogma shift in the 1950s and changed the understanding of tissue repair[7]. These ground-breaking seminal studies by Abercrombie et al. paved the way for the discovery of the myofibroblast in the early 1970s by Gabbiani et al. in transmission electron micrographs of wound granulation tissue of healing injuries in rats[8]. The authors described wound fibroblasts that exhibited a "fibrillar system developed within the cytoplasm", which "were no longer the few fibrils often seen in fibroblasts, but bundles of packed fibrils resembling those of smooth muscle"[8]. In a subsequent functional study, the same authors concluded that "fibroblasts, under certain conditions, are capable of modulating towards a cell type that is structurally and functionally close to smooth muscle; for these cells the name 'myofibroblast' may be appropriate"[9]. In this Review, we provide and revisit definitions of different cells with a fibroblast phenotype in the adult organism in their normal and active state, one of which is the myofibroblast.

Transient activation of fibroblastic cells into myofibroblasts is crucial and beneficial to repair acute tissue damage whereas their persistent activation characterizes and drives organ scarring, a debilitating and often lethal condition called fibrosis[10] (Fig. 1a). Why myofibroblasts persist in fibrosis but disappear following acute repair is not entirely clear, and solving this conundrum is considered the holy grail in fibrosis research. For deeper insight into the specific contributions of myofibroblasts in pathological settings, the reader is referred to excellent recent reviews on the fibrosis of different organs and tissues, including the heart[11,12], kidneys[13], liver[14], intestine[15,16], central nervous system[17], lungs[18], skin[10,19], palmar fascia (that is, the triangular connective tissue structure in the palm of the hand)[20] and eyes[21,22]. In addition to their molecular identity, the local environment is crucial to determining the function and fate of fibroblastic cells in physiological and pathological tissue repair. Here, we will mainly elaborate on the impact of the mechanical (tissue stiffness and strain) and biochemical (growth factors and ECM ligands) environment in promoting fibroblastic cell activation, function and survival. We present how continued exposure to these factors creates epigenetic memory that turns transient myofibroblasts into persistent myofibroblasts. Different concepts of therapeutic interference with the respective fibroblast-specific signalling recognition and transduction mechanisms are presented, with the aim of devising strategies to improve the outcomes of dysregulated healing.

Fibroblast functions and phenotypes

We now understand that multiple different cell types, including fibroblasts, can become activated into myofibroblasts after acute tissue injury, which is a particularly efficient strategy used by the body to rapidly repair structural damage[10] (Fig. 1a), irrespective of where in the body the damage occurs. The vast majority of myofibroblast precursors are of mesenchymal origin although some reports suggest that epithelial cells[23,24] (through epithelial–mesenchymal transition), vascular endothelial cells (by endothelial–mesenchymal transition)[25], and circulating CD45-positive cells of the haematopoietic lineage, such as fibrocytes and macrophages[26,27], can contribute to the myofibroblast pool under specific conditions and in a tissue-specific manner. For instance, in the central nervous system, astrocytes and glial cells, in addition to pericytes, can attain the myofibroblast phenotype and function to produce collagen scars[17,28]. The most common mesenchymal myofibroblast precursors include adipocytes, pericytes, smooth muscle cells (SMCs), multipotent mesenchymal stromal or stem cells (MSCs)[29–31], and bona fide fibroblasts[19,29,32,33]. In the following section, we will try to untangle some of the confusing fibroblast–myofibroblast nomenclature. It should be emphasized that the myofibroblast is a phenotype that many different precursors can acquire to perform repair functions and not a distinct cell type.

Fibroblast and myofibroblast heterogeneity

That fibroblasts are a vastly heterogeneous group has long been recognized, even in the pre-single-cell RNA-sequencing (scRNA-seq) era[34]. If anything, one commonality of fibroblastic cells is the difficulty to define unique molecular markers — hence their original denomination as fibroplastic[3]. There seems to be general agreement that fibroblastic cells in most normal tissues (that is, not activated) express collagen type I[35] and the platelet-derived growth factor (PDGF) receptor-α (PDGFRα)[36]. Other frequently used fibroblast markers are vimentin, hypermethylated in cancer 1 (Hic1)[37,38], CD90 (Thy1)[39], transcription factor 21 (TCF21)[30] and fibroblast-specific protein 1 (FSP1; also known as S100A4)[40]. However, many of these mesenchymal markers are also expressed in SMCs and pericytes and are thus not specific to fibroblasts. In contrast to fibroblasts, pericytes and SMCs both express desmin, and (non-activated) pericytes are positive for chondroitin sulfate proteoglycan 4 (NG2) and PDGFRβ but not PDGFRα[41–43]. Exclusion criteria (for example, being positive for mesenchymal vimentin, but negative for both the muscle marker desmin and the endothelial cell marker CD31) can be employed to discriminate fibroblastic cells from other common tissue cell types using conventional immunohistochemistry[44] (Box 1).

Another prominent group of mesenchymal myofibroblast precursors easily confounded with fibroblasts are MSCs, where even the letters in the acronym are a matter of debate, with 'S' standing for 'stromal' or 'stem', and 'M' meaning mesenchymal or multipotent[45–47]. MSCs together with fibro-adipogenic progenitors and pericytes often reside in a perivascular niche[29]. With their long projections extending along vessels, perivascular cells are ideally positioned to sense

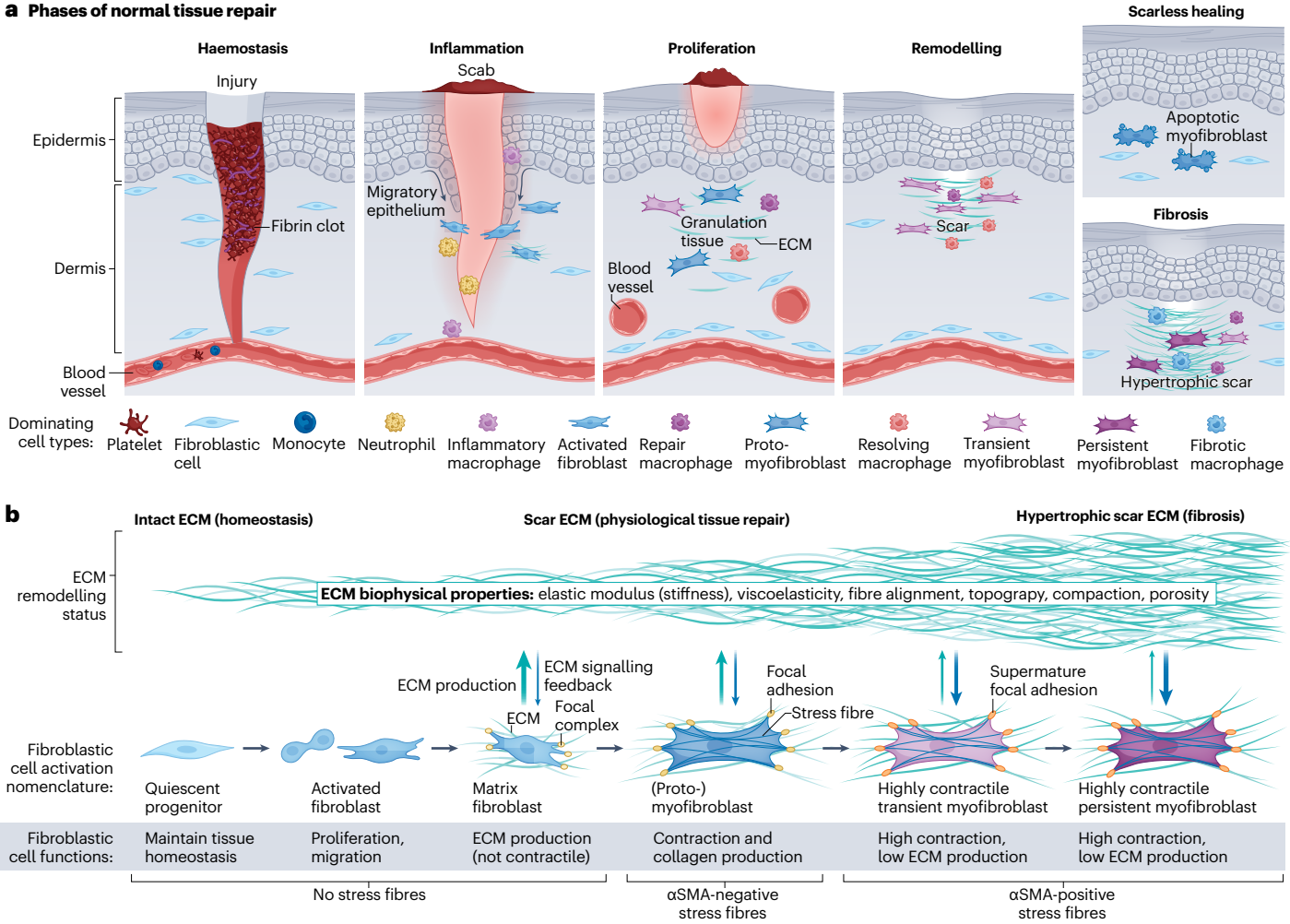


Fig. 1 | Myofibroblast activation states in normal and pathological tissue repair. This figure illustrates the different stages of the tissue repair process and the involvement of different cell types after an injury to the skin but, in principle, these processes apply to any organ injury. **a**, During haemostasis, bleeding is stopped by a platelet-initiated fibrin clot that also serves as a scaffold for growth factors and cells that populate the early wound bed. This is followed by inflammation involving early-responding mast cells, neutrophils and inflammatory macrophages that fight infections and phagocytose debris and secrete factors that coordinate the healing process. The proliferation phase is characterized by neovascularization, recruitment of repair macrophages and activation of resident cells in the adjacent normal tissue to divide and migrate into the wound. Organs with epithelium are re-epithelialized and fibroblastic cells migrate into the provisional extracellular matrix (ECM) to produce collagen, which is further aligned and organized into scar tissue in the remodelling phase. Resolving macrophages contribute to the volume reduction of the wound bed by removing excess ECM. In physiological repair, macrophages and myofibroblasts are eventually cleared by apoptosis, when the original structure and function of normal tissue are largely restored, allowing scarless healing. In fibrotic repair, contractile myofibroblasts and repair macrophages persist and continue to drive scar ECM production and stiffening, leading to hypertrophic scars. **b**, Fibroblast activation into contractile myofibroblasts is tightly controlled by the organization state (that is, the biophysical properties) of the wound ECM and vice versa. Starting in the inflammatory phase, fibroblastic cells are activated from a homeostatic 'quiescent' progenitor state to proliferate and migrate into the provisional fibrin-rich ECM. The low traction forces of migrating fibroblasts initially result in the alignment and later in the condensation of fibrin fibrils. Fibrin-rich ECM is progressively replaced by fibrous collagens produced by initially non-contractile matrix fibroblasts in the proliferation phase. Once the denser and stiffer collagen ECM becomes too resistant to low fibroblast traction forces, it stimulates fibroblast-mediated formation of contractile actomyosin bundles (so-called stress fibres) in later wound stages. The higher contractile forces of such (proto-)myofibroblasts produce even stiffer scar ECM that can withstand external forces and restores the mechanical integrity of the organ. To match this higher mechanical resistance, myofibroblasts start to express α-smooth muscle actin (αSMA), the incorporation of which into stress fibres hallmarks the highly contractile myofibroblast. Along with enhanced extracellular and intracellular stress, cell–ECM adhesions develop from so-called immature focal complexes into mature focal adhesions and then into the supermature focal adhesions typical for αSMA-positive myofibroblasts. Maturation refers to the gradually increasing size and molecular complexity of myofibroblast adhesion sites. As a consequence, myofibroblasts generate the mechanical conditions that stimulate and maintain their contractile phenotype in a positive feedback loop. Stress fibre-rich, αSMA-expressing myofibroblasts were formerly referred to as differentiated; however, this may not be accurate given that they can revert into stress fibre-negative cells after withdrawal of mechanical stress and pro-fibrotic signalling factors. Therefore, the distinction should instead be made between persistent myofibroblasts that prevail in organ fibrosis and transient myofibroblasts that are cleared by apoptosis during physiological repair. Identifying the mechanisms that discriminate one from the other is among the biggest challenges in fibrosis research.

Box 1

Myofibroblast detection and characterization

For the diagnosis and grading of fibroproliferative diseases, it is important to identify myofibroblasts in histological sections of biopsies, but how myofibroblasts are best characterized in pathology and research applications remains an important question. Bundles of actin microfilaments, although sufficient for (proto-)myofibroblast identification, cannot be specifically detected in standard histological procedures because the filamentous actin-binding probe phalloidin is incompatible with the organic solvents used in the process[44]. Detection of α-smooth muscle actin (αSMA) with actin isoform-specific antibodies is the most widely used method to identify myofibroblasts in cell culture and sections of fibroproliferative tissues[306]. Other markers that are characteristic of the myofibroblast cytoskeleton in various fibrotic organs and conditions are the transmembrane receptors α11β1 integrin[307], cadherin 11 (also known as OB-cadherin)[305,308,309], platelet-derived growth factor receptor-β (PDGFRβ)[42,153] and, possibly, leucine-rich repeat-containing 15 (LRRC15)[53]. Extracellular matrix proteins associated with myofibroblasts include extradomain A (ED-A) fibronectin[133], periostin[310], tenascin C[311], latent transforming growth factor-β-binding protein 1 (LTBP1)[155,163], CCN2 (formerly called connective tissue growth factor; CTGF)[312] and, determined only recently, the protein collagen triple helix repeat-containing 1 (CTHRC1)[56,313]. Proteomic and spatial proteomic approaches will likely expand the list of extracellular matrix-associated myofibroblast markers[314,315]. The expression levels and thus usefulness of these proteins as myofibroblast markers vary depending on tissue and condition. All these frequently used markers, including αSMA-positive stress fibres, are upregulated during myofibroblast activation from various precursors but are not exclusive to myofibroblasts. For instance, αSMA is expressed in contractile bundles of smooth muscle cells (SMCs) and myoepithelial cells and as a cytoplasmic protein (that is, not in stress fibres) in pericytes[316,317]. ED-A fibronectin is also expressed by vascular SMCs[165] and cadherin 11 is upregulated in fibroblast-like synoviocytes in rheumatoid diseases[318], that is, it is also characteristic of immune fibroblasts[119].

Combinatorial assessments with exclusion markers are often required to discriminate myofibroblasts from phenotypically related cells that occupy similar spaces in relevant tissues. SMCs, as one of the most prominent confounders, express specific muscle markers such as desmin, smoothelin and smooth muscle myosin that are absent from myofibroblasts[44]. Other proteins that are typically not expressed by myofibroblasts but are by their non-activated precursors are PDGFRα[36] and, in specific conditions, Thy1 membrane glycoprotein. The latter has a functional role in regulating mechanical fibroblast–myofibroblast initial activation but was reported to be absent from later myofibroblast activation stages[319]. Whether Thy1 is indicative of myofibroblasts (for example, in scleroderma skin)[320] or absent from myofibroblasts in fibrotic lesions (for example, lungs and heart)[39,321] seems to vary across different fibrotic organs. In cancer-associated fibroblasts, high expression of Thy1 is indicative of cancer progression in contrast to Thy1-negative cancer-associated fibroblasts, which indicate earlier stages of cancer development[322]. Understanding of the role of Thy1 is further confounded by the fact that mRNA signatures do not necessarily inform functional phenotype. Thy1 mRNA signatures are often diffusely spread across fibroblast subpopulations annotated from single-cell RNA sequencing datasets. However, the expression or lack of Thy1 on the surface of fibroblastic cells consistently yields discrete functional phenotypes that are clearly not captured in uniform manifold approximation and projection or t-distributed stochastic neighbour embedding plots of single-cell RNA sequencing but are rather due to the molecular function of Thy1 protein. In fact, Thy1 and other proteins whose transcriptional states do not align with annotated fibroblast subpopulations but confer clear functional phenotypes may present a new axis in the further discrimination of fibroblast and myofibroblast functions.

environmental changes and are among the first responders to tissue damage and danger signals[29]. Because these cells also reside in the adventitial connective tissue layer of larger vessels they are sometimes called fibroblasts. However, multipotent perivascular 'mural cells' should be treated distinctly from adventitial fibroblasts as the latter lack progenitor potential, that is, the ability to differentiate into multiple different cell types[48–50]. As for specific markers, plasticity is a virtue of MSCs[47]. To name an example, fibro-adipogenic progenitors are considered MSCs because they can differentiate into (at least) fibroblasts and adipocytes in skeletal and heart muscle; in these tissues, they express characteristic markers such as PDGFRα, Sca1, Hic1 and Gli1 (refs. 37,51,52).

Fibroblastic progenitor cell populations with similar proposed functions have been described for most organs, and aligning their annotation with molecular marker sets across organs is ongoing[50,53] (Box 1). For instance, different fibroblast subpopulations have been characterized in the skin according to their location in the adipose, facial, reticular and papillary layers, function, and characteristic protein and transcription profiles[38,54]. Likewise, lung fibroblasts have been classified as airway-associated alveolar fibroblasts, lipofibroblasts and matrix fibroblasts with distinct predicted functions and locations[55–59]. At steady state, that is, in normal (mouse) tissues, careful curation of publicly available and experimentally generated scRNA-seq datasets identified common gene signatures of PDGFRα-sorted fibroblasts in all organs, including the expression of *Pi16* (encoding peptidase inhibitor 16), *Col15a1* (encoding collagen α1(XV) chain) and *Dpt* (encoding dermatopontin)[53]. In contrast to other tissue-specific fibroblast-associated genes (Box 1), *Dpt* emerges as a 'universal' tissue fibroblast marker, and its expression level is inversely correlated with fibroblast specialization for tissue-specific functions[53]. *Dpt*-positive fibroblasts also give rise to *Lrrc15*-positive (encoding leucine-rich repeat-containing 15 (LRRC15)) fibroblasts, which are prominent myofibroblast precursors in different injury conditions, including cancer[53,60].

One potential confounder of scRNA-seq data is that distinct clusters in uniform manifold approximation and projection or t-distributed stochastic neighbour embedding plots (Box 2) are interpreted as

differentiated fibroblast types but may merely reflect the transcriptional profiles related to their current function – that is, they may not be terminally differentiated. In the case of fully differentiated fibroblasts, transition from one into another fibroblast type should not be possible. However, MSCs and fibroblastic cells have the amazing capacity to adapt phenotypically, functionally and transcriptionally – not only to the different environments that exist across tissues but also to the niches within the same normal adult organs[50]. It exceeds the scope of this Review to list all the different fibroblast states or types with their respective marker sets and the reader is referred to reviews covering fibroblast heterogeneity specifically in the lungs[61], skin[33], heart[62], kidneys[13], liver[63] and intestine[15].

Most of these populations can become activated into myofibroblasts, yet with different quantitative contributions to the overall myofibroblast pool depending on their location and the severity and chronicity of organ injury. One possible exception is so-called immune or inflammatory fibroblasts that seem to be activated from the same perivascular precursor cells after tissue damage, like myofibroblasts[64], but then follow a different path (Box 3). Fibroblastic cells with immune cell functions also exist in normal tissues – for example, the specialized fibroblastic reticular cells that support immune B and T cells in lymphoid organs[65,66], and the fibroblast-like synoviocytes that line the joints[67]. The proposed dichotomy between fibroblastic cells with either immune (cytokine secretion) or myofibroblast (ECM secretion and contraction) functions is best studied for cancer-associated fibroblasts that form and populate the scar-like stroma around tumours[68,69]. Recent non-peer-reviewed computational predictions suggest that inflammatory fibroblasts could be an intermediate precursor to ECM-producing fibroblasts in mouse and human lungs[70]. Likewise, CD201-positive progenitor cells in subcutaneous fascia were shown to contribute to mouse dermal wound healing by passing through a 'pro-inflammatory' state before acquiring myofibroblast features and functions[71]. Thus, whether immune fibroblasts and myofibroblasts are sequential or distinct states remains an open question. We will next consider the myofibroblast activation state responsible for the functional aspects of tissue repair.

Myofibroblasts

The ability to exert contractile force is, quite literally, part of the myofibroblast identity and the functional definition of these cells. Cytoskeletal actin–myosin bundles in vivo are the structural prerequisite for contractile function and the defining morphological feature of myofibroblasts (Fig. 1b and Box 1). Simply put, cells that do not contract or are missing contractile cytoskeletal elements cannot be and should not be labelled myofibroblasts[72]. This is an important consideration, as fibroblastic cells will adopt different functions during different stages of tissue repair, such as enhanced proliferation, migration, cytokine and ECM secretion, ECM remodelling, and phagocytosis[10] (Fig. 1b). Because these functions and the related transcriptional profiles are acquired de novo after injury, the underlying process is typically referred to as 'fibroblast activation'. All these different fibroblast activation states are critical for proper tissue repair but alone do not meet the myofibroblast criterion unless contractile features and function are present.

In standard cell culture conditions, that is, on plastic dishes in the presence of serum, all cultured fibroblastic cells form actin–myosin bundles, called stress fibres in vitro, and are therefore technically myofibroblasts. To emphasize this fact, we previously introduced the term 'proto-myofibroblast' as a pre-activation state of highly contractile (at the time called 'differentiated') myofibroblasts, and both of these activation states coexist in cell cultures[5]. The stress fibres of highly contractile myofibroblasts are distinct from those of proto-myofibroblasts by additionally containing the actin isoform α-smooth muscle actin (αSMA)[73] (Fig. 1b). In vivo formation of actin filament bundles precedes and is likely a pre-condition for the stress fibre incorporation of αSMA during skin wound healing[74]. Incorporation of αSMA into pre-existing β-cytoplasmic actin filament bundles enhances the contractile activity of stress fibres[73], which can be abolished by pharmacological removal of αSMA from stress fibres using competitive peptides[75]. It is still unclear how αSMA confers higher contractile activity than other actin isoforms at similar expression levels and how αSMA recruitment to stress fibres is controlled at the molecular level. It is conceivable that the contribution of specific tropomyosin isoforms[76] and/or actin isoform-specific filament nucleation factors of the formin family[77] is responsible for the differing functions. It remains to be shown if some of these 'accessory'

Box 2

Single-cell approaches to the study of fibroblasts

Over the past years, single-cell RNA sequencing (scRNA-seq) has become a powerful tool with the potential to transform fibroblast research and enhance understanding of lineage relationships between these enigmatic cells. When interpreting uniform manifold approximation and projection or t-distributed stochastic neighbour embedding plots of scRNA-seq data we need to be aware that clusters are typically produced in an unbiased fashion based on gene expression similarities whereas the annotation of the clusters is not unbiased. For example, the widely used public databases CellMarker 2.0 and CancerSEA[323,324] are manually curated and rely on the accuracy of studies that contributed to the formulation of fibroblast definition criteria. Even unbiased annotation creates self-reinforcing biases based on what is known, or believed to be known, from pre-existing literature and the specific interpretation of the literature by those defining what constitutes distinct and different subpopulations. Users of such databases should be aware that genes such as *ACTA2* (encoding α-smooth muscle actin (αSMA)) and *DES* (encoding desmin) appear high in fibroblast annotation lists but are hardly suitable fibroblast markers, since they are not normally expressed in fibroblasts. Likewise, *DES* and *MYH11* (encoding smooth muscle myosin heavy chain) are listed as myofibroblast markers, but they are not typically expressed in myofibroblasts. Other confounding factors of scRNA-seq datasets are inconsistent isolation procedures of fibroblastic cells: as these cells are usually embedded in dense connective tissues, enrichment of fibroblasts for subsequent analysis often uses the expression of platelet-derived growth factor receptor-α (PDGFRα)[53] or type I collagen as sorting criteria[56]. Such inclusion and exclusion criteria will necessarily result in bias, although experimental or computational cell sorting are currently required to manage clustering algorithms that demand significant computational resources. As technology progresses, such biases will become less of an issue.

Box 3

Immune functions of fibroblasts

Fibroblastic cells are increasingly shown to acquire bona fide immune functions in response to injury and danger signals, such as loss of extracellular matrix (ECM) and vascular integrity and inflammation. Whether such immune fibroblasts represent a distinct fibroblast type that is mutually exclusive with ECM-producing and contracting functions of myofibroblasts, or whether both functional states can transition into each other, is a matter of ongoing debate. Confirming earlier reports of IL-1-activated immune cancer-associated fibroblasts (CAFs) and transforming growth factor-β1 (TGFβ1)-activated myofibroblastic 'myCAFs'[325], these distinct CAF subpopulations have recently been expanded by single-cell RNA sequencing-based pseudotime lineage tracing in pancreatic cancer[326]. The study supported the notion that TGFβ1-driven, leucine-rich repeat-containing 15 (LRRC15)-positive CAFs with structural support functions and IL-1-driven LRRC15-negative CAFs with immunoregulatory functions derive from two distinct 'resting' fibroblasts populations. However, a later study suggested a shared trajectory with a common, *Pi16*-positive root precursor state that becomes activated via *Col15a1*-positive intermediates[53].

The mechanisms that mediate the ability of fibroblastic cells to detect and communicate tissue damage overlap the molecular damage sensors used by innate immune cells. For instance, fibroblasts and professional immune cells both rely on the same families of danger signal receptors, including Toll-like receptors (TLRs), intracellular nucleotide-binding oligomerization domain containing 1, IL-1 receptor (IL-1R) and receptors for advanced glycation end products[119]. Advanced glycation end products are the products of non-enzymatic reactions between reducing sugars with proteins, lipids or nucleic acids that accumulate with age and other fibrosis risk factors such as diabetes. These commonalities suggest that some fibroblasts, like inflammatory cells, can directly sense damage-associated molecular patterns (DAMPs). DAMPs released from necrotic myocardial cells in the heart were shown to directly activate fibroblasts in vitro[327]. In vivo, however, it has been difficult to assess whether the various DAMPs and alarmins released after tissue damage act directly on fibroblasts or do so, for example, by first triggering inflammatory cells to produce cytokines that activate fibroblasts.

There is strong evidence of fibroblastic cell activation, including cytokine expression, in the absence of inflammatory infiltrates from central nervous system injury models of denervation, as well as in patients suffering from amyotrophic lateral sclerosis[328]. That fibroblasts may indeed be the first responders to tissue damage and initiate inflammation is further supported by the fact that cytokine production, including of chemotactic factors for inflammatory cells, is among their initial functions following specification. Following tissue injury, fibroblastic cells exhibit highly upregulated levels of chemokines known to recruit type 1, early response immune cells from the circulation such as C-C motif chemokine 2 (CCL2; also known as monocyte chemoattractant protein 1), which is a main signal for monocyte extravasation[329], C-X-C motif chemokine ligand 14, which amplifies chemotactic responses, and CCL5, an attractant for neutrophils[330]. Fibroblastic cells also release cytokines associated with type 2 responses, such as CCL11, an eosinophil-attracting chemokine, and IL-33, supporting type 2 innate lymphoid cells and T regulatory cells[331,332]. The recruited and tissue-resident inflammatory cells then produce multiple cytokines that can, in turn, act on fibroblasts, including TGFβ, IL-4, IL-13, hepatocyte growth factor (HGF), IGF1 and IL-1 (ref. 287).

The IL-1R signalling pathway is likely to have a central role in this circuit between immune fibroblasts and inflammatory cells. Ubiquitous deletion of IL-1R leads to a reduction in both inflammatory cells and cytokines, as well as myofibroblast formation and ECM deposition in the ischaemic heart[333]. IL-1α treatment of isolated cardiac fibroblasts in vitro directly induces the expression of inflammatory cytokines, such as tumour necrosis factor (TNF), IL-6 and IL-1β[334], all of which act on inflammatory cells, suggesting that IL-1α elicits a fibroblast-driven positive feedback on inflammatory cell function. Thus, a fibroblast-initiated self-reinforcing circuit with inflammatory cells seems to be established following tissue damage, where the full inflammatory response is only achieved when both cell systems are engaged by DAMPs, potentially providing multiple layers of control against inappropriate activation of a regenerative environment that fosters tissue repair.

proteins bear value as myofibroblast molecular markers as suggested for palladin, a protein that is expressed in different isoforms, some of which are specifically found in myofibroblast stress fibres[78,79]. Other criteria to characterize myofibroblasts and discriminate them from other cell types are summarized in Box 1.

Proliferation, migration, ECM production, formation of contractile stress fibres, expression of αSMA and incorporation of αSMA into existing stress fibres are generally viewed as sequential but overlapping features of individual fibroblastic cells (Fig. 1b). However, it is also conceivable that different fibroblast precursor subpopulations (or lineages) specialize differently according to the tissue repair context, like the immune fibroblasts discussed above and in Box 3, and as described for different subpopulations of dermal and fascial fibroblasts during skin wound healing[80]. Furthermore, it is plausible that fibroblast functional specialization occurs at the expense of versatility and that highly contractile myofibroblasts may no longer be proficient at ECM secretion (or never were because they did not pass through an ECM-producing state). Distinct fibroblast lineages and/or specialization stages can explain the separation of 'matrix fibroblasts' (a tautology, really, because fibroblasts are ECM-producing cells and thus by definition reside in the matrix) and contractile myofibroblasts, for instance, in scRNA-seq clusters generated from fibroblast profiles in developing and fibrotic lungs[56,81,82]. Although not consistently tested in experimental models, it is conceivable that upregulation of contractile activity negatively feeds back on ECM production, and vice versa. After all, contraction is only meaningful if there is a substrate to contract. Functional specialization can also explain why lung fibroblasts experimentally sorted for high collagen promoter activity and

expression exhibit no or only low levels of the contractile marker αSMA[56]. It remains to be shown whether these populations have contractile capacity by forming αSMA-negative stress fibres. However, this question cannot be answered by assessing mRNA or using standard (solvent-based) histological processing alone (Box 2). Whether the proposed functional specialization states of fibroblasts are transient (as in normal repair) or more permanent (as has been suggested for fibrosis) will be discussed in our later section on myofibroblast fates.

Myofibroblast activation

The mechanisms of myofibroblast activation appear remarkably similar across different pathophysiological conditions of tissue injury and repair despite the heterogeneity of their precursor cells, as discussed above. In the following sections, we focus on mechanical factors because the contraction of fibrous collagen ECM by myofibroblasts results in tissue stiffening, which in turn enhances myofibroblast activation. Breaking this positive feedback loop is one of the most challenging, but also most promising, strategies for targeting myofibroblasts in anti-fibrotic or pro-healing therapies. We will first describe key biochemical signals, that is, cytokines, growth factors and ECM-associated molecules (Fig. 2), which are also pursued as anti-fibrotic targets and are crucial for the understanding of subsequent sections.

Biochemical signals regulating myofibroblast activation

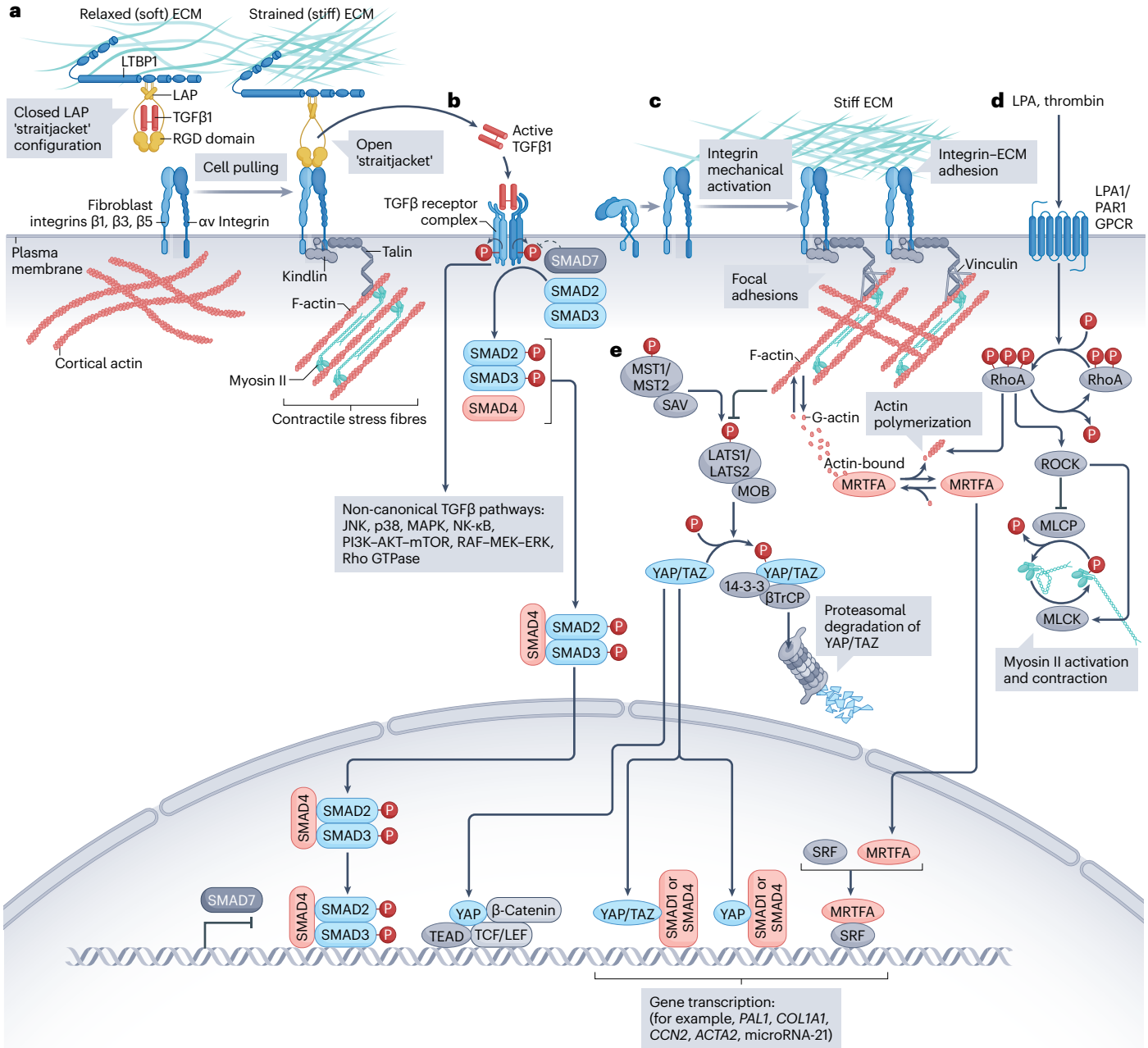
Transforming growth factor-β1 (TGFβ1) is one of the first, best-described and most potent cytokines known to induce myofibroblast activation[83]. Because extracellular activation of TGFβ1, which is kept inactive in complex with its latency-associated pro-peptide (LAP) (Fig. 2a), by fibroblastic cells has a mechanical component, it will be discussed below under mechanotransduction mechanisms for myofibroblast activation. In addition to TGFβ1, other TGFβ family members, including activin, TGFβ2 and TGFβ3, have all been shown to drive myofibroblast activation once liberated from their respective LAPs by assembling and binding to the same TGFβ receptor[84]. The transmembrane TGFβ receptor complex is formed by heterodimerization of the TGFβ receptor type I (Alk5; also known as TGFR1) and the TGFβ type II receptor (TGFR2)[85–87]. Its signalling can be enhanced by associating with co-receptors such as endoglin (CD105) or the TGFβ type III receptor (betaglycan; also known as TGFR3). Canonical TGFβ signalling is mediated through serine and/or threonine phosphorylation of the receptor-regulated SMAD proteins SMAD2 and SMAD3, which form a complex with SMAD4 after phosphorylation that can translocate to the nucleus (Fig. 2b). Binding of the SMAD2–SMAD3–SMAD4 complex to SMAD-binding elements in the promoter of pro-fibrotic genes such as the gene encoding αSMA enhances their transcription[85–87].

The TGFβ1 pathway is neither strictly linear nor does it act in isolation but rather contributes to a complex signalling network. The SMAD2–SMAD3–SMAD4 complex also acts as a co-transcription factor together with other factors such as Yes-associated protein 1 (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ, together referred to as YAP/TAZ), myocardin-related transcription factor A (MRTFA; also known as MKL)[88], as well as T cell factor (TCF) and Wnt signalling-related lymphoid enhancer factor (LEF)[89]. Jointly, they promote the transcription of myofibroblast-associated genes such as those encoding αSMA and collagen type I. In addition to canonical SMAD-dependent signalling, tyrosine kinase activities of the TGFβ receptor complex also act non-canonically through MAPK–ERK, RAF–MEK1/2–ERK1/2, p38 MAPK, Jun kinase, NF-κB, PI3K–AKT–mTOR, and Rho–Rho-associated kinase (ROCK)[90,91] to drive the expression of ECM and/or contraction-related genes (Fig. 2b). The ROCK, YAP/TAZ and MRTFA pathways will be discussed below in the context of myofibroblast mechanosignalling. Beyond TGFβ1, numerous biochemical factors and their receptors have been studied for their function in controlling myofibroblast activation – often from the perspective of their potential use as therapeutic targets. Most prominently considered are IL-4, IL-6, IL-11, IL-13, angiotensin II, endothelin 1, platelet-derived growth factor (PDGF), lysophosphatidic acid receptor 1 and TLR4 (refs. 92–95).

Mechanical myofibroblast activation

Many cell or tissue culture and animal experimental models (Supplementary Boxes S1–S3) of tissue injury and fibrosis are based on the activation of myofibroblasts by mechanical stress[96] (Supplementary Tables S1 and S2). Among various mechanical factors, the stress exerted by the 'stiffness' of wound and fibrotic ECM is a major driver of myofibroblast activation[72,97,98]. Atomic force microscopy is the most widely used technique to measure the elastic moduli (in pascal)[99] of different normal human and rodent organs, including skin (0.1–10 kPa), brain (0. 1–0.5 kPa), fat tissue (1–3 kPa), lungs (0.5–3 kPa), liver (1–2 kPa), kidney, striated skeletal and heart muscles (10–15 kPa), and the stiff tissues building teeth and bone (1–4 GPa)[100]. An overview of the moduli measured for these normal and fibrotic organs is provided in an excellent recent review[100]. The changes in ECM stiffness that occur after injury are key signals for fibroblasts to adopt their activity and activation state to the progress of repair, which is best studied in skin healing[10,101]. When the protective ECM architecture is lost after injury, external strains and stresses are directly transmitted to tissue-resident cells. Although not proven in vivo, it is tempting to speculate that the initial loss of structural integrity in damaged tissues is a mechanical trigger for migratory and ECM secretion programmes in fibroblastic cells residing at the wound edge. This idea is consistent with the concept of tensional homeostasis, that is, the observation that fibroblasts re-establish their baseline tensional states by contracting after experimental relaxation or by relaxing following experimental stretching in 3D collagen ECM[5,102]. In addition to changes in mechanical stress, physical damage may generate ECM fragments and epitopes that serve as danger signals to activate fibroblasts and as biomarkers for clinicians[103–106].

Before these pre-activated resident fibroblasts are activated and migrate into the wound bed, the wound gap is typically filled with a platelet-rich, fibrin-rich ECM[10,107] (Fig. 1a). This fibrin clot provides a 'sticky' reservoir for growth factors of platelets that release their cytoplasmic content after exposure to ECM, and rapidly stops bleeding if vessels have lost integrity. From a mechanical point of view, fibrin-rich ECM is initially approximately ten times softer than normal skin, with an elastic modulus of 0.1–1.0 kPa (ref. 108) (softer than cooked glass noodles, which curiously are much softer than overcooked spaghetti) but undergoes substantial strain stiffening through the contraction of activated platelets[109]. Thus, fibrin-rich ECM not only provides a scaffold for fibroblasts to migrate into the wound bed but also affords the mechanical resistance required for the initiation of fibroblast contractile features (that is, formation of stress fibres). Whereas the provisional fibrin-rich ECM starts to be degraded shortly after its formation, fibroblasts begin to restore and maintain tissue integrity within a few days by producing an ECM rich in cellular fibronectin, as well as type III and type I collagen[10]. The low pulling forces of migratory fibroblasts and proto-myofibroblasts gradually stiffen the ECM to reach elastic moduli of ~20 kPa (similar to a marshmallow). Initial ECM stiffening is further promoted by inflammatory cell-mediated fibre crosslinking[110];



curiously, the release of ECM fibre tension (for example, after injury) seems to favour the recruitment of crosslinking enzymes such as tissue-resident transglutaminases[111]. These processes accumulate over time and higher contractile forces are required to further remodel the now more mature scar; at this mechanical checkpoint, collagen-producing and low-contractile proto-myofibroblasts transition to the highly contractile αSMA-positive myofibroblasts[44] (Fig. 1b).

Transient ECM stiffening is required to protect wound tissue from rupture, but the dysregulated healing of untreated large-area wounds and/or chronic myofibroblast activation inevitably result in contractures. In skin, the stiffness of fibrotic (hypertrophic) scar ECM resulting from continued myofibroblast activities can reach elastic moduli of 50–100 kPa, that is, a hundred times stiffer than normal skin[112] (almost as stiff as a middle-aged gouda cheese). Increases in stiffness by one to two orders of magnitude are also reported for the fibrotic ECM accumulating in other organs[100]. ECM stiffness is further enhanced through chemical crosslinks established by fibroblastic and inflammatory cell-derived transglutaminases[113], lysyl hydroxylases[114], lysyl oxidases[115] and lysyl oxidase-like enzymes[116]. Consequently, fibrotic ECM is not only highly dense but also more resistant to resolution by proteolytic enzymes[117,118]. In addition to obstructing the function of the affected organ (for example, lung expansion, heart pumping, kidney blood filtration), stiff fibrotic scar tissue further drives the progression of fibrosis by turning healthy precursor cells into fibrotic myofibroblasts, thereby establishing a self-perpetuating fibrotic feedback loop[101,119]. It is one of the most daunting tasks and currently unreached goals of

Fig. 2 | Signalling pathways regulating myofibroblast activation. **a**, Fibroblasts secrete transforming growth factor-β1 (TGFβ1) in complex with its latency-associated peptide (LAP); this latent complex can be stored in the extracellular matrix (ECM) by covalent binding to latent TGFβ1-binding protein 1 (LTBP1). Within the mechanically resistant ECM characteristic of tissues under repair, cell contractile forces transmitted through intracellular actomyosin bundles (stress fibres) and αv integrins to arginine–glycine–aspartate (RGD)-binding domains in LAP induce a conformational change in the latent TGFβ1 'straitjacket' conformation that releases active TGFβ1. **b**, Active TGFβ1 binds to and assembles the TGFβ receptor complex and promotes canonical SMAD signalling, resulting in the translocation of SMAD4–SMAD2–SMAD3 complexes to the nucleus, where they regulate the transcription of genes such as *ACTA2* (encoding α-smooth muscle actin). This action is counteracted by the negative regulator SMAD7. In addition, TGFβ1 binding initiates non-canonical signalling through JNK, p38, ERK, MAPK and Rho–Rho-associated kinase (ROCK) signalling. **c**, Extracellular binding of integrins in the presence of mechanical stress (for example, stiff ECM) results in an integrin conformational change, which starts a recruitment cascade of structural and signalling proteins to the cytoplasmic portion of integrins, resulting in the formation of adhesion complexes. Focal adhesion proteins involved in fibroblast mechanotransduction include the focal adhesion kinase (FAK; not shown), vinculin, kindlin and talin, which mediate binding to actin filaments (F-actin) and facilitate the formation of stress fibres. **d**, Biochemical and biomechanical myofibroblast signalling pathways intersect at multiple junctures and create a complex signalling network in which the state of actin polymerization takes centre stage. In addition to being a downstream target of TGFβ1 receptor signalling, ROCK is activated by the GTP-bound form of RhoA following the binding of lysophosphatidic acid (LPA) and thrombin to G protein-coupled receptors (GPCR). A key downstream event of ROCK activation is enhanced fibroblast contraction via inhibition of myosin light chain phosphatase (MLCP) and activation of myosin light chain kinase (MLCK). Rho GTPases also directly affect actin polymerization by regulating actin monomer-binding and polymer-binding proteins (not shown here). The status of actin polymerization further controls gene transcription through regulation of mechanosensitive co-transcription factors such as myocardin-related transcription factor A (MRTFA). Actin polymerization results in the release of MRTFA from globular (G)-actin and allows MRTFA translocation into the nucleus. Association of nuclear MRTFA with serum response factor (SRF) drives the transcription of pro-fibrotic gene products such as *CCN2* and *ACTA2*. **e**, In addition, actin polymerization and contraction control gene expression by regulating the nuclear localization of the transcriptional co-activator with PDZ-binding motif (TAZ) and Yes-associated protein (YAP). For instance, RhoA activation and subsequent formation of stress fibres leads to the inhibition of large tumour suppressor homologue 1 (LATS1) and LATS2 in the cytosol. LATS1 and LATS2 are part of the Hippo signalling pathway and downstream targets of the mammalian STE20-like kinase 1 (MST1), MST2 and the scaffolding protein SAV. Inhibition of LATS1 and LATS2 increases the fraction of cytosolic unphosphorylated YAP and TAZ. Phosphorylated YAP and TAZ are prone to proteasomal degradation in complex with protein 14-3-3 and the ubiquitin ligase βTrCP. In contrast, non-phosphorylated YAP and TAZ translocate into the nucleus where they associate with co-transcription factors such as TEA domain family of DNA-binding proteins (TEAD), β-catenin and the T cell factor–lymphoid enhancing factor (TCF–LEF) to drive the transcription of pro-fibrotic genes such as *CCN2* and microRNA-21. Note that transcription factor-binding sites are not drawn in the correct sequence and not all transcription factors are required at the same time to drive pro-fibrotic gene expression. PAR1, proteinase-activated receptor 1.

anti-fibrotic therapies to break this loop, which has evolved multiple redundancies to promote rapid healing of injured tissues at the expense of functionality[72]. Attractive targets for anti-fibrotic strategies are found in the molecular mechanisms that closely tie myofibroblast activation to their mechanical environment. This includes cell components that sense ECM mechanics and transduce the generated signals into myofibroblast-promoting transcription programmes. Other potential therapeutic targets are presented by the ECM in a stress-dependent manner, including cryptic recognition sites that open after strain for the binding of cell receptors and other ECM ligands but remain hidden under homeostatic conditions. Another ECM stiffness-dependent and strain-dependent event in the myofibroblast activation cascade is the release of active TGFβ1 from ECM latent stores. Mechanical activation of TGFβ1 and revelation of cryptic ECM domains will be discussed in the next sections.

Mechanisms of (myo-)fibroblast mechanoperception

Fibroblastic cells can sense and respond to mechanical cues emanating from the extracellular milieu through different cell surface receptors such as integrins, discoidin domain receptors (DDR), G protein-coupled receptors (GPCR) and stretch-activated ion channels (SACs)[120]. All these receptor systems activate mechanotransduction signalling pathways[120] that ultimately regulate fibroblast activation states either acutely, for instance by promoting the polymerization of actin into filaments and stress fibres, or at the transcription level.

ECM receptors of fibroblasts and myofibroblasts

Fibroblastic cells transmit forces to contract ECM into scar tissue and probe the chemical and mechanical properties of their environment by physically linking their contractile cytoskeleton to transmembrane ECM adhesion receptor complexes[121]. The most prominent fibroblast ECM receptors include the hyaluronic acid receptor CD44, DDR1 and DDR2, and various integrins. Hyaluronic acid binding to CD44 induces the formation of adhesion complexes of adaptor proteins that link to the contractile actin cytoskeleton[122]. Activation of CD44 and its downstream effectors are critical to fibroblast motility and invasion in response to stiffening ECM and blockage of CD44 abrogates stiff remodelling in the fibrotic lung and post-infarct heart[123].

DDR1 has increasingly attracted the attention of researchers due to its specific binding and compaction of fibrillar collagen in a mechanically regulated and myosin-mediated process[124]. Expression of DDR1 on fibroblastic cells is enhanced in tissues affected by fibroproliferative disorders, including cancer, skin hypertrophic scars, liver, kidney and lung fibrosis, and it is thus considered a drug target[125,126]. Analogously, integrin β1 can bind to different α-integrins to form α1β1, α2β1, α10β1 and α11β1 heterodimers, all of which have been described as fibroblast collagen receptors involved in tissue repair, for instance, of skin and heart[98,127]. Because of their enriched, if not exclusive, expression on mesenchymal myofibroblast precursor cells, integrins α11β1 and α1β8 are being scrutinized in experimental animal models for their potential to inhibit fibrosis in the liver, lungs and cancer stroma[128–130]. Functional deletion of integrin α11β1, but not of the other collagen receptor integrins α1β1 and α2β1, resulted in reduced fibroblast activation and reduced skin scarring[131]. Despite showing some specificity for fibroblastic cells, integrin α1β8 can bind multiple ECM ligands and its pro-fibrotic mode of action is just beginning to be unravelled[130].

Part of the binding promiscuity of integrin α1β8 is due to its recognition of the arginine–glycine–aspartate (RGD) motif, which is present in multiple ECM proteins such as in vitronectin and fibronectin, which

contribute to the formation of provisional ECM after injury, and in periostin, thrombospondins and fibrillins, which are enriched in fibrotic ECM[132,133]. Accordingly, other RGD-recognizing integrins, such as α5β1, αvβ3 and αvβ5, were also shown to mediate fibroblast mechanosensing, force transmission and myofibroblast activation[134–136]. However, their potential as specific myofibroblast targets is limited by their expression on multiple other, unrelated cell types. The RGD binding motif is also a key recognition site found in the latent TGFβ1–LAP complex. Here, the RGD motif mediates high-affinity binding to integrins αvβ1 (mainly found on fibroblastic cells)[137], αvβ6 (only epithelial cells)[138] and αvβ8 (mainly immune cells)[139], all of which were shown to activate latent TGFβ1 in the extracellular space in vitro and in animal models of fibrosis[85,86] (Fig. 2a). How fibroblastic cells use contractile force to activate latent TGFβ1 held in complex with LAP in the ECM will be covered in the next section after discussing the role of mechanical stress in enhancing fibroblast adhesion as a prerequisite for myofibroblast activation.

Fibroblasts contract stronger on stiffer substrates in an attempt to maintain tensional homeostasis, that is, equilibrium between ECM resistance and intracellular force[140]. Consequently, the strength of their adhesions must be adapted to withstand higher stresses. Limiting adhesion maturation by restricting the substrate surfaces available for fibroblast attachment, substrate adhesiveness or stiffness are used as experimental strategies to reduce intracellular stress and myofibroblast activation[135]. For example, patterning of body implants with adhesive micro-islands has been explored as an approach to prevent myofibroblast activation and fibrosis at the surface of body implants[141]. Mechanical stress directly regulates the binding affinity of fibroblastic integrins by a strain-induced conformational change in the β-integrin subunit, which initiates a cascade of intracellular signalling events that culminate in the maturation of adhesion sites in terms of size and strength from small focal complexes into mature focal adhesions and supermature adhesions in myofibroblasts[121] (Fig. 1b). Supermature focal adhesions have been described as ECM adhesions 10–20 µm in length specific to αSMA-positive cultured myofibroblasts[142]. Compared to the 2–6-µm long focal adhesions of αSMA-negative proto-myofibroblasts, they can sustain higher stress, that is, force per area, and thus allow the formation of highly contractile intracellular stress fibres[135]. Structures equivalent to supermature adhesions, called fibronexus, have been described in different tissue-residing myofibroblasts, first in the granulation tissue of healing guinea pig skin wounds[143].

Among the hundreds of molecular components that are recruited during the maturation of integrin adhesions, talin and kindlin family members take centre stage by directly binding to integrin tails and the actin cytoskeleton in a stress-dependent manner[144,145] (Fig. 2). Kindlin 2 expression is elevated in experimental models of kidney, lung, heart and skin fibrosis, and functional loss of kindlin 2 prevents mechanical activation of fibroblasts cultured from these tissues[146,147]. Focal adhesion kinase (FAK) and integrin-linked kinase (ILK; which, ironically, has no kinase activity and does not bind to integrins) are other mechanosensitive key components of fibroblast adhesions. Experimentally induced loss of ILK reduces fibroblast force transduction, TGFβ1 secretion, myofibroblast activation and myofibroblast-mediated tissue contraction in mouse skin and kidney fibrosis, possibly by loss of focal adhesion structure[148,149]. Likewise, loss of FAK function in cultured fibroblasts reduces myofibroblast activation[150] and thereby alleviates fibrotic features in mouse models of hypertrophic scarring[151]. Given that these signalling and structural components are expressed in all adhesive cells, targeting their function with general inhibitors rather than fibroblast-specific genetic knockout bears the risk of side effects. Higher specificity can be expected for the targeting of fibroblast-specific integrins that are differentially expressed in tissue repair conditions, such as integrin α11β1, and has been shown for the αv integrins involved in fibroblast-mediated activation of latent TGFβ1 (refs. 152,153), which we discuss next.

Mechanochemical crosstalk in myofibroblast activation

Although typically thought of as a soluble cue that exhibits its effects through cell–cell paracrine signalling, activation of TGFβ is intimately tied to the mechanical state of the ECM and fibroblasts. Fibroblastic cells secrete latent TGFβ1 in complex with its pro-peptide LAP. In contrast to the pro-peptides of other secreted factors that are typically cleaved off during secretion, LAP is cleaved intracellularly by furin enzymes but remains non-covalently associated with the TGFβ1 cytokine after secretion. This association traps the TGFβ1 cytokine in an inactive 'straitjacket' configuration[154]. LAP covalently binds to the fibrillin protein family member latent TGFβ1-binding protein 1 (LTBP1), thereby enabling long-term storage of latent TGFβ1 in the ECM[155]. Other cells, notably T regulatory cells of the adaptive immune system, use the same covalent binding domain in LAP to store latent TGFβ1 in the extracellular space but, instead of using LTBP1, they link LAP to the membrane proteins TGFβ activators LRRC32 or LRRC33 (refs. 156,157). Several publications have shown and reviewed how different cell types use different mechanisms of latent TGFβ1 presentation and activation, thereby achieving precise control over the local TGFβ1 availability[85,86,158]. For ECM-bound latent TGFβ1, the integrins αvβ1, αvβ3, αvβ5 and αvβ6 have all been demonstrated to mechanically activate latent TGFβ1 by transmitting pulling forces to the RGD domain in LAP[152,153] (Fig. 2a,b). Consequently, specific inhibition or genetic knockout of the latent TGFβ1-binding fibroblast integrins successfully inhibits the development of fibrosis in different experimental animal models[137,138,153,159,160]. Unlike the LAP of TGFβ1, the latent pro-peptide of TGFβ2, another pro-fibrotic actor and myofibroblast-activating cytokine, lacks the αv integrin–RGD binding site[161,162]. It remains unclear whether integrins have a role in TGFβ2 activation, but recent studies support the hypothesis that αvβ6 integrins on activated lung epithelial cells bind to a specialized binding motif in pro-TGFβ2 and contribute to TGFβ2 activation in a strain-dependent process[161,162]. Cell-pulling forces exercised on the latent TGFβ1–LTBP1 complex must meet a mechanically resisting, that is, sufficiently pre-strained and remodelled, ECM to be able to induce a conformational change in the LAP–TGFβ1 straitjacket configuration and to liberate the active TGFβ1 cytokine[163]. Notably, the (in vitro) stiffness enabling TGFβ1 activation by fibroblasts is lower (~3 kPa)[152] than that required for αSMA expression in stress fibres (~16 kPa)[135]. Therefore, mechanical activation of TGFβ1 serves as a first mechanical checkpoint for full myofibroblast activation.

Structural changes in ECM proteins under strain often reveal new protein motifs that function as signals for fibroblastic cells in the mechanical conditions of tissue under repair but are hidden when no strain is applied. For example, fibronectin has a domain structure perfectly adapted to orchestrate complex repair scenarios. It serves as a hub for multiple cytokines and growth factors and as an initial structural template to guide the assembly of other ECM proteins, including collagens, fibrillins and LTBP1 (ref. 164). Differential splicing of cellular fibronectin produces the extradomain (type III) variants ED-B and ED-A fibronectin[164,165]. The latter is produced only in fibroproliferative conditions, is a prerequisite molecule for myofibroblast activation[166], and presents binding sites for the integrins α4β7, α4β1 and α9β1 (ref. 133). Other 'cryptic' binding epitopes outside the

extradomain variants may become accessible only in the presence of ED-A and/or ED-B, as proposed for LTBP1 binding[167]. Notably, cryptic fibronectin self-assembly and signalling sites are revealed after mechanical strain[168,169] — a feature that can promote myofibroblast activation even in a soft environment[134]. Primary lung fibroblasts cultured on very soft (~680 Pa) fibronectin-rich ECM display increased spreading, contractility and nuclear translocation of the canonical myofibroblast transcription factor myocardin-related transcription factor A (MRTFA)[134]. This myofibroblast-promoting mechanoresponse is in stark contrast to the expectation of myofibroblast suppression on soft substrates and can be explained by a strain-induced conformational change in the central cell integrin receptor-binding region of fibronectin. Applying strain to fibronectin is proposed to prompt an 'integrin mechano-switch' from α5β1 integrin to αvβ3 integrin binding in this domain[134]. Elevated binding of integrin αvβ3 then allows myofibroblast activation in what would otherwise be considered a mechanically impermissible environment[134]. Biophysical analysis of fibronectin has demonstrated that, after relaxation of mechanical forces, such as those generated by activated fibroblasts, it is capable of fully recovering its original conformation[170,171], thus flipping the switch back towards α5β1 integrin binding. It is tempting to speculate that force-mediated conformational changes in fibronectin, like the integrin mechano-switch, are leveraged during normal tissue repair to allow fast adaptations of fibroblast activities to acute and local changes in the mechanical environment.

Myofibroblast stretch-activated channels

Enhanced mechanical strain is also the basis for the activation of SACs localized to the fibroblast membrane to coordinate myofibroblast activation and activity in response to extracellular stress[172,173]. The SAC transient receptor potential cation channel subfamily V member 4 (TRPV4) co-localizes with both integrin β1 and DDR1 adhesions, where it regulates fibroblast ECM remodelling activities by controlling the local influx of Ca^{2+} ions[174,175]. TRPV4 is also important for stress-mediated and TGFβ1-mediated myofibroblast activation of skin, lung and cardiac fibroblasts, and loss of TRPV4 function protects mouse hearts from experimentally induced fibrosis[176–178]. Similarly, myofibroblast activation of dermal fibroblasts is regulated by TRPV3 in a process dependent on SMAD2 and SMAD3 in vitro and in vivo[179]. One decade after the discovery of Piezo1 and Piezo2 as key components of another class of SACs[180], a role for Piezo1 in regulating fibroblast repair functions is beginning to emerge. Mechanical stimulation of Piezo1 elicits downstream signalling that culminates in myofibroblast activation in skin hypertrophic scarring[181], kidney fibrosis[182] and heart fibrosis[183]. Like TRPV4, Piezo1 has been found in adhesions, where it contributes to fibroblast sensing of ECM stiffness[184]. SACs of yet unresolved molecular composition are further involved in coordinating the contractile activities of small groups of myofibroblasts that are physically connected by actin-associated adherens junctions[185]. In cell–cell and cell–ECM adhesions, SACs are ideally positioned to adapt fibroblast-remodelling activities in close feedback with the mechanical state of the ECM and their neighbouring cells by coordinating Ca^{2+} influx, downstream Rho-associated protein kinase (ROCK) signalling and actin–myosin activity[186].

Mechanisms of myofibroblast mechanotransduction

So far, we have discussed how fibroblastic cells perceive mechanical signals and how initial biochemical signals, such as active TGFβ1 or high cytosolic Ca^{2+}, arise from mechanical events. Before establishing the link between myofibroblast mechanosensing and their activation at the level of transcription, we will provide a brief overview of how the same signals acutely regulate myofibroblast contractile activity in response to changes in the mechanical environment. More details on the regulation of fibroblastic cell contraction can be obtained from our recent review specifically on the topic[72].

Calcium, Rho, ROCK and myofibroblast contraction

Myofibroblast contraction is principally controlled by myosin light chain (MLC) kinase (MLCK)-mediated phosphorylation of the myosin light chains (MLCs) associated with non-muscle myosin 2 (MYH2)[187,188]. Phosphorylated myosin heads bind to actin filaments within stress fibres and produce force in the form of a power stroke, not unlike the contraction of the much higher organized sarcomeres of muscle cells[72,189] (Fig. 2). Conversely, de-phosphorylation of MLC by the MLC phosphatase (MLCP) results in myosin unbinding from actin filaments, which terminates the contraction cycle[72,189]. Myofibroblasts exhibit at least two modes of contraction that are regulated by either enhancing MLCK activity through Ca^{2+} signalling or inhibiting MLCP activity through RhoA–ROCK signalling[187,188] (Fig. 2d). We previously proposed a lock-step model in which both contraction modes act in concert to locally release ECM fibres from stress to allow their shortening while maintaining overall ECM integrity[5]. According to this model, RhoA–ROCK signalling controls longer-lasting (hours), long-ranging (micrometre) and strong (micro-Newtons) contractile tone that creates and maintains slack in ECM fibres against a tense environment. Stress-released ECM fibres are then amenable to Ca^{2+}-mediated contractions that are smaller in range (nanometre) and force (nano-Newtons) but occur regularly and last a few minutes each[187]. Contractile amplitude and frequency are acutely adapted to increasing extracellular resistance by enhancing the frequency of Ca^{2+} oscillations and RhoA activities[190,191]. The transiently contracted ECM state is proposed to be established by proteolytic processing and crosslinking of collagen fibres–at this point, the load can now be transferred from the contracted myofibroblast to the shortened ECM, and myofibroblasts can re-spread for a new cycle. This hypothesis is supported by experimental observations that collagen fibres are protected from proteolytic cleavage in collagenase-rich environments when under an experimental mechanical load but dissolve when relaxed[117].

Of the three Rho isoforms, RhoA is best studied in the context of myofibroblast activation, whereas the roles of RhoB and RhoC are yet to be resolved[192]. Likewise, ROCK1 and ROCK2 both control myofibroblast contractile force and activation in mechanically stressed environments and are considered potential therapeutic targets in lung and heart fibrosis[193–195]. However, given that Ca^{2+}, Rho and ROCK control many cellular processes and regulate the physiological contraction of other cell types in fibrotic organs (for example, cardiomyocytes in the heart, airway SMCs in lungs and vascular SMCs in all organs), specific intervention with myofibroblast contraction should rather be implemented upstream of these central signalling pathways. For instance, ligand binding to the G protein-coupled receptors (GPCR) lysophosphatidic acid (LPA) receptor 1 or proteinase-activated receptor 1 (PAR1) has been described to contribute to myofibroblast activation and enhanced contraction through RhoA–ROCK[196–198]. The associated G proteins that initiate pro-fibrotic signalling cascades are $G_{\alpha i/o}$, $G_{\alpha q11}$, $G_{\alpha 12/13}$ and $G_{\alpha s}$ (refs. 162,199). Conversely, the oestrogen GPCR and the G protein $G_{\alpha s}$ were shown to have anti-fibrotic functions and can be stimulated to suppress myofibroblast activation in experimental

Glossary

Arginine–glycine–aspartate (RGD) motif
The RGD amino acid sequence is a motif in the latent transforming growth factor-β1 complex and many proteins of the extracellular matrix, where it promotes specific binding of αv integrins; the presence of an adjacent 'synergy sequence' in fibronectin also allows integrins αIIbβ3 and α5β1 to bind to RGD.

Granulation tissue
This provisional tissue fills wounds during healing, is rich in various cells and small vessels, and appears in histological cross-sections as 'granulose'.

Pericytes
Also referred to as perivascular cells, pericytes show fibroblast-like morphology, are closely associated with endothelial cells of the microvasculature and often have progenitor capacity, that is, the ability to differentiate into multiple cell types, including fibroblasts.

Stiffness
Most biological studies dealing with cell, extracellular matrix or tissue 'stiffness' refer to the elastic modulus (that is, stress over strain) in pascal, which is a relatively accurate parameter when measured at the length scale of cells and below.

Strain stiffening
In contrast to ideally elastic synthetic polymers, biological polymers such as collagen, fibrin and cytoskeletal filaments get stiffer when they are extended (that is, strained). Strain stiffening protects tissues from overstraining and enables the fibrous collagen around large blood vessels to undergo small peristaltic movements but to cuff the vessel under high blood pressure.

Stress fibres
Contractile bundles composed of actin and myosin filaments as part of the cytoskeleton that are typically formed in response to mechanical stress and often terminate in cell adhesions, which connect to the extracellular matrix or other cells.

Tropomyosin
Tropomyosins are filamentous (F-) actin-binding proteins that regulate actin–myosin interactions in adult striated and smooth muscle, as well as in non-muscle contractile cells such as myofibroblasts.

models of fibrosis[200]. Notably, these signalling functions of GPCRs can relate to ROCK-mediated contraction but also intersect with other pathways, such as JAK2–STAT3 (ref. 201), nuclear factor of activated T cells (NFAT)[202] and YAP/TAZ signalling[199], to regulate myofibroblast activation at the level of transcription.

YAP, TAZ and YAP/TAZ

YAP and TAZ were originally discovered as members of the Hippo pathway that can become activated by various upstream stimuli, including those relevant for myofibroblast activation and fibrosis such as Wnt and GPCR ligands[203]. The Hippo signalling pathway consists of a cascade of mammalian STE20-like kinase 1 (MST1; also known as serine–threonine-protein kinase 4), MST2, large tumour suppressor homologue 1 (LATS1) and LATS2 (Fig. 2e). The phosphorylated and thus activated serine–threonine kinases LATS1 and LATS2 phosphorylate YAP and TAZ, resulting in their cytoplasmic sequestration and/or proteasomal degradation[203]. In addition to their role in Hippo signalling, YAP and TAZ have become widely known for their role as mechanosensitive transcription factors. Both localize to the nucleus of fibroblasts grown on fibrosis-stiff culture substrates and under mechanical stress but remain in the cytosol in fibroblasts grown on physiologically soft culture substrates[204] (Supplementary Box S1 and Supplementary Table S1). Activation of RhoA and subsequent formation of stress fibres leads to the inhibition of LATS1 and LATS2 in the cytoplasm and enhances the pool of cytosolic unphosphorylated YAP and TAZ[205]. Unphosphorylated YAP and TAZ can translocate into the nucleus and regulate target gene expression through interactions with members of the TEA domain family of DNA-binding proteins (TEAD), as well as with SMADs, p73 and Runt-related transcription factor 1 (RUNX), to mediate transcription of fibrosis-related genes. Among the YAP and TAZ target genes are *CCN2* (mentioned above) and microRNA-21, which will be discussed further below in the context of myofibroblast memory[206].

Sustained nuclear activity of YAP and TAZ drives transcription of ECM-associated and contraction-associated genes in myofibroblasts of the kidney, lung, liver and palmar fascia[207]. Early activation of YAP/TAZ during tissue repair may serve to prime fibroblasts to respond to TGFβ signals as nuclear YAP/TAZ promotes SMAD2 and SMAD3 nuclear retainment[208]. The same studies demonstrated that inhibition of YAP/TAZ signalling reduces experimentally induced organ fibrosis. Although often considered as a pair, YAP and TAZ have both redundant and independent roles and, whereas single knockout of either factor reduces fibroblast activation, dual knockout induces a significantly more potent effect[209,210]. Although the full mechanism remains to be elucidated, the redundancy observed from single knockouts of YAP or TAZ may in part be mediated by compensatory activities of other TEAD-binding co-factors in the nucleus. In addition to the above-described regulation of YAP and TAZ nuclear translocation, pro-fibrotic signals such as TGFβ1 (via SMAD3) can also regulate the expression levels — at least of TAZ — during myofibroblast activation. Curiously, regulation of TAZ transcription is dependent on another mechanosensitive co-transcription factor, MRTFA[211].

Myocardin-related transcription factor A

Analogous to YAP and TAZ, MRTFA translocates from the cytosol, where it is sequestered by binding to non-polymerized globular actin monomers, into the nucleus of mechanically stressed cells (Fig. 2). Once released from sequestration after stress-induced actin polymerization in the cytosol, MRTFA acts as a co-transcription factor in combination with serum response factor (SRF) to increase target gene transcription[211]. MRTF–SRF target genes include the myofibroblast-associated genes *CCN2* and *ACTA2* (encoding αSMA) and genes encoding stress-sensing and stress-transmitting cytoskeletal components, such as the integrins α2 and α11 (collagen binding), integrin αv (fibronectin and latent TGFβ1 binding) and the corresponding β-integrins β1, β3 and β5, as well as integrin-linked protein kinase (ILK) and TAZ[148]. MRTFA was first discovered as a driver of myofibroblast activation in cultured kidney tubular epithelial cells, where it contributes to epithelial–mesenchymal transition and expression of αSMA in response to TGFβ1 and injury[212]. Later, the same function was confirmed in various fibroblastic cells in conditions mimicking physiological and pathological repair[213]. Consequently, treatment of dermal wounds with the MRTFA agonist isoxazole increases the accumulation of αSMA-positive cells in granulation tissue and thereby accelerates normal wound closure[214]. Conversely, second-generation pharmacological inhibitors of MRTF–SRF signalling, such as the compounds CCG-222740, CCG-100602 and CCG-203971, protect against cancer-associated pancreatic fibrosis[215] and intestinal[216] and eye[217] fibrosis, and mice with fibroblasts deficient in MRTFA and MRTFB are protected from renal fibrosis[148]. Exciting recent studies have started to establish a link between acute induction of myofibroblast transcription programmes by MRTFA and YAP/TAZ

and lasting myofibroblast activation by epigenetic reprogramming as discussed in the next section[218].

Myofibroblast fates and memory

One of the central questions in fibrosis research is whether myofibroblasts in fibrotic lesions are fundamentally different to those in normally healing tissues. The authors of this Review argue that they are principally the same, that is, activated by the same molecular and mechanical mechanisms from the same precursor cells. However, myofibroblasts disappear at the end of normal tissue repair, that is, they are transient, but persist in fibrosis[219] (Fig. 1a). In the following section, we will discuss how prolonged and continued exposure to a fibrotic environment generates epigenetic memory that converts transient myofibroblasts into persistent myofibroblasts. Initially, myofibroblasts were considered to represent a terminally 'differentiated' fibroblast state and apoptosis was thought to be their ultimate fate[219]. However, it is now evident that myofibroblast deactivation and switching between different fibroblast activation states is possible, including reversal of the highly activated αSMA-positive myofibroblast phenotype to proto-myofibroblasts or stress fibre-negative fibroblastic cells[220]. Deactivation seems to be the prominent myofibroblast clearance mechanism during liver repair[35], whereas myofibroblast apoptosis prevails in repaired skin wounds[221], as in most organs. Both apoptosis and necroptosis have been described to reduce the numbers of cardiac myofibroblasts and both processes have been experimentally targeted in vivo to improve heart repair after injury[222,223]. We and others have reviewed how myofibroblast survival is regulated by their acquired resistance to death and how this resistance is controlled by mechanical factors[219,224]. Here, we will focus on the regulation of myofibroblast fate through epigenetic modifications, that is, heritable but reversible phenotypic alterations mediated by non-coding RNAs or more stable modifications at the level of the DNA and histones. Epigenetic imprinting and maintenance of myofibroblast features have long been recognized as a reason for myofibroblast persistence in fibrosis[225,226] but myofibroblast epigenetic control through mechanical factors is a comparably young and unexplored field of research.

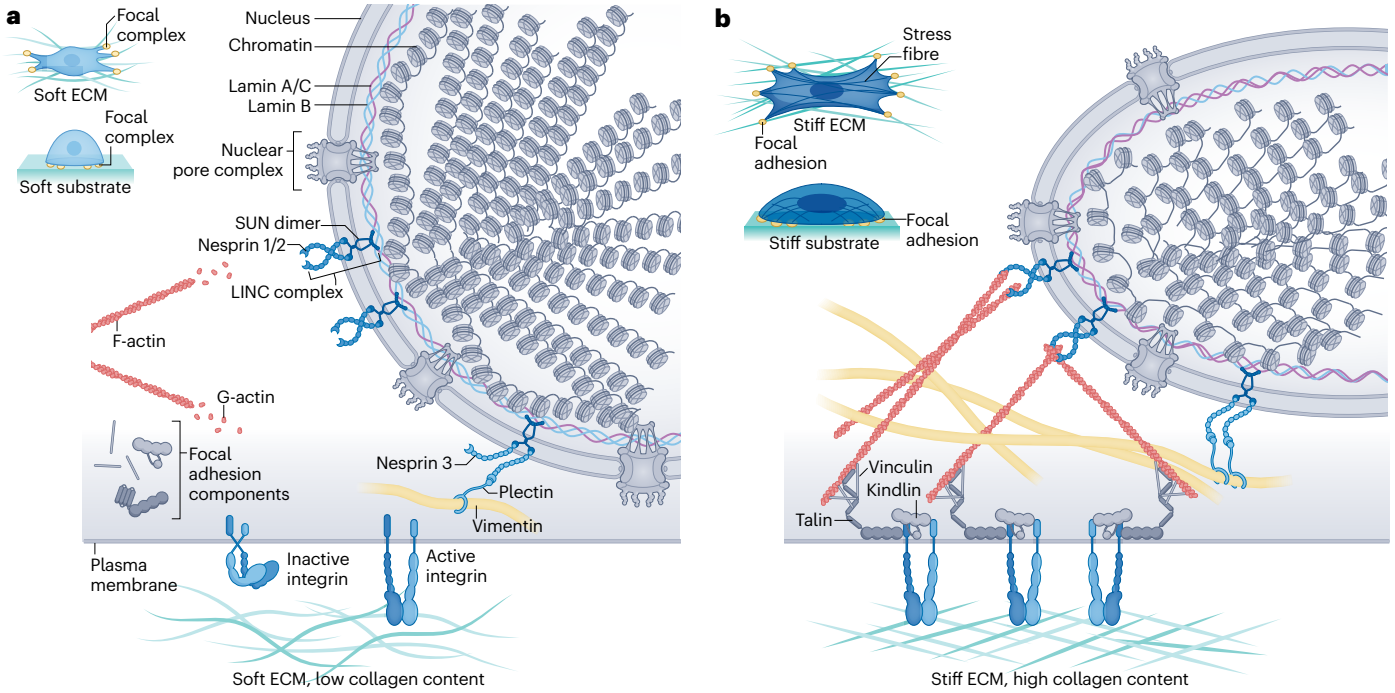
Mechanical memory promotes myofibroblast persistence

Earlier work has discovered that prolonged exposure, or 'priming', of lung fibroblasts and MSCs to pathophysiologically stiff and soft culture substrates imprints myofibroblast activation states that persist even after the switch to a different mechanical environment[227,228]. We accordingly coined the term 'mechanical memory'. The duration of mechanical priming (hours–weeks) and the strength of the mechanical stimulus (dosing), for example, the magnitude of the substrate elastic modulus, affect the duration of memory. Notably, there is no consensus on the values of elastic moduli to label substrates as 'soft' or 'stiff' — both are used relative to each other and to the normal physiological environment of the cells; here, we align with the definitions given in the respective studies. For guidance on the elastic moduli measured for normal (approximately 0.1–5 kPa) and fibrotic connective tissues (>10 kPa), see our section on mechanical myofibroblast activation above.

Mechanical memory was later reported to also stabilize bone and fat lineage choices of MSCs on stiff and soft substrates[229] and MSCs subjected to dynamic tensile loading on nanofibrous scaffolds[230]. MSCs are frequently chosen as a model to study mechanical regulation of cell fate, which includes MSC–myofibroblast activation, either as a pre-state of osteogenesis or as a fibrogenic lineage choice[231]. Concomitantly, mechanical memory of prior experience with a stiff culture environment was shown to maintain the myofibroblast activation state of other cells of MSC lineage such as chondrocytes[232], which reduces their suitability for subsequent grafting to heal destroyed cartilage. Mechanical reprogramming was proposed to 'rejuvenate' mesenchymal cells, that is, restoring their potential to repair damaged tissues instead of producing adverse scar tissue[233]. It seems that mechanical stress hierarchically engages acute mechanotransduction first and later promotes epigenetic modifications to generate memory depending on the duration (dose) and intensity (amplitude) of the stimulus[234]. For instance, mechanical priming of human MSCs for 3–10 days on stiff surfaces (10 kPa) generates transient memory maintained by YAP-mediated gene expression programmes[229]. In contrast, 'stiff-priming' (100 kPa) for 3 weeks establishes high levels of microRNA-21, which drive pro-fibrotic programmes for 2 weeks following the removal of mechanical stress[228]. The duration of mechanical memory in these studies likely depended on the retention time of YAP in the nucleus (hours) and the turnover rates of microRNA-21 (days).

Exposure to a stiff mechanical environment can imprint even longer-lasting, yet reversible, epigenetic modifications made at the level of histones and DNA. Repeated tensile loading of bovine MSCs resulted in enlarged cell nuclei and decreased chromatin condensation[235]. How long these features are maintained after mechanical unloading depends on the duration, number and amplitude of the priming loading cycles and on the histone methyltransferase (MET) EZH2 (ref. 235). Human and mouse lung fibroblasts cultured on stiff polymer (64-kPa) and stiff plastic (gigapascal) substrates show higher methylation levels of histone H3 at lysine residue K9 (H3K9me) than those of fibroblasts cultured on soft (0.4-kPa) substrates[236]. Human MSCs grown for 3 days on stiff substrates (33 kPa) have larger nuclei, lower chromatin condensation, higher expression of histone deacetylases (HDACs) and lower expression of histone acetyltransferases (HATs) than that of soft-grown MSCs (5.5 kPa)[237]; later, these changes were shown to be associated with higher histone acetylation and MSC osteogenesis[238]. Expression levels of the epigenetic modifiers HDAC and HAT adapt to new mechanical environments after only 1 day of priming but stabilize after 10 days of priming[237]. The same authors reported myofibroblast memory acquired by porcine heart valve fibroblasts, primed on a moderately stiff (4-kPa) substrate for 2–7 days and then switched to a softer substrate (2 kPa) for 2 days[239,240]. In these experiments, maintenance of myofibroblast features after stiff–soft switch also depended on the priming duration. Stiff-grown fibroblasts exhibited specific open chromatin regions that contained binding motifs for transcription factors driving mechanosensing pathways and myofibroblast gene expression, including αSMA[239]. Global chromatin condensation was found to acutely (1 day) decrease after exposure to stiff substrates but then gradually increase over time (3–9 days); this observation was explained with nuclear stiffening in response to higher stress on resistant substrates[239]. Indeed, there is a linear scaling relation between collagen content (and thus, ECM stiffness) and lamin A contents in the nuclear envelope across different stages of fibrosis, indicating that ECM conditions directly reflect nuclear mechanics and pro-fibrotic signalling[241].

Changes in the chromatin state of cells cultured in different mechanical environments are mediated by a direct link between substrate and nuclear mechanics[242] (Fig. 3a,b). Critical for extracellular–intranuclear force transmission from substrate adhesions are intact actin and intermediate filaments — vimentin in the case of fibroblasts — and their interaction with the linker of nucleoskeleton


a
Focal complex
Soft ECM
Focal complex
Soft substrate
Nucleus
Chromatin
Lamin A/C
Lamin B
Nuclear pore complex
SUN dimer
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LINC complex
F-actin
G-actin
Focal adhesion components
Nesprin 3
Plectin
Vimentin
Plasma membrane
Inactive integrin
Active integrin
Soft ECM, low collagen content
b
Stress fibre
Stiff ECM
Focal adhesion
Stiff substrate
Focal adhesion
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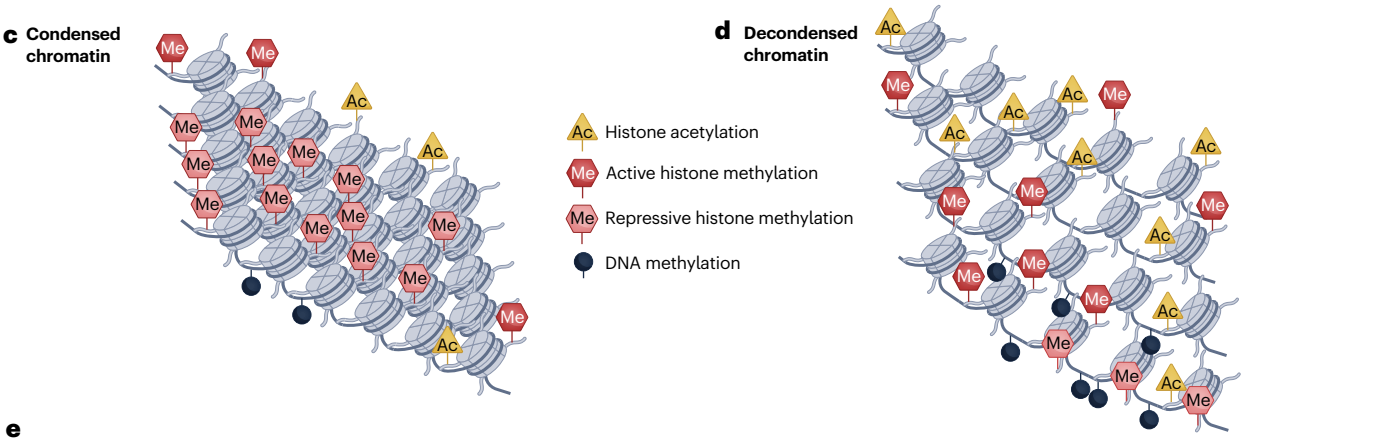
Kindlin
Talin
Stiff ECM, high collagen content


c Condensed chromatin
d Decondensed chromatin
Ac Histone acetylation
Me Active histone methylation
Me Repressive histone methylation
DNA methylation

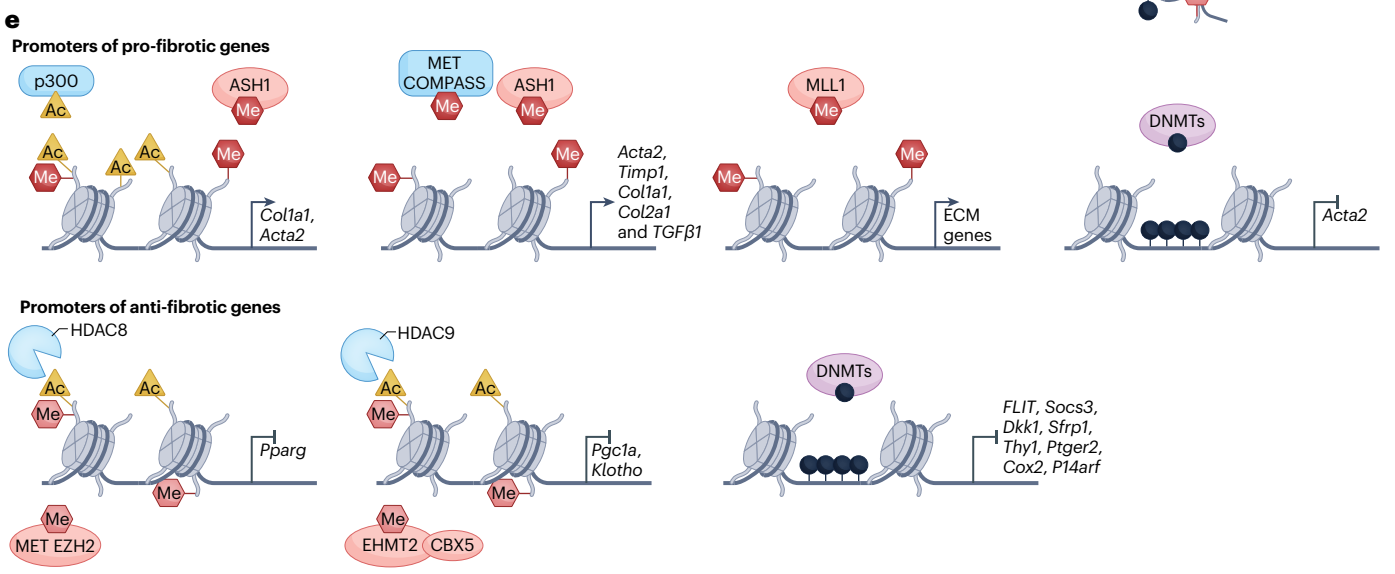

e
Promoters of pro-fibrotic genes
p300
ASH1
Col1a1, Acta2
MET COMPASS
ASH1
Acta2, Timp1, Col1a1, Col2a1 and TGFβ1
MLL1
ECM genes
DNMTs
Acta2
Promoters of anti-fibrotic genes
HDAC8
Pparg
MET EZH2
HDAC9
Pgc1a, Klotho
EHMT2
CBX5
DNMTs
FLIT, Socs3, Dkk1, Sfrp1, Thy1, Ptger2, Cox2, P14arf

Fig. 3 | Nuclear mechanotransduction and myofibroblast memory. a, The integrin-containing focal adhesions of fibroblasts adhering to soft extracellular matrix (ECM) are immature, that is, they lack molecular components that could facilitate the organization of cytoskeletal actin microfilaments into stress fibres. Such relaxed fibroblasts are also characterized by a low degree of polymerization of the intermediate filament vimentin, low lamin A to lamin C ratios in the inner nuclear membrane, and overall condensed chromatin. **b**, A stiff ECM provides high resistance, which enables the maturation of focal adhesions and promotes signalling processes that result in actin polymerization from globular (G-actin) to filamentous (F-actin) actin and the formation of stress fibres and, thus, lead to higher intracellular stress. F-actin stress fibres can establish a direct mechanical connection between ECM adhesions and chromatin in the cell nucleus by binding to the nuclear envelope spectrin repeat protein 1 (nesprin 1) and nesprin 2. Nesprins, in turn, bind to Sad1p and UNC-84 homology (SUN) proteins that span the nuclear membrane and, in the inner nuclear membrane, interact with lamin A, which attaches to chromatin. The complex of SUN proteins and nesprins in the nuclear outer membrane is called linker of nucleoskeleton and cytoskeleton (LINC) complex. Analogous to the connection of nesprin 1 and nesprin 2 with F-actin, vimentin filaments connect to nesprin 3. In both cases, transmission of stress from cytoskeletal filaments to the LINC complex forces opening of chromatin, thereby changing DNA accessibility for transcriptional and epigenetic regulations. The lamin A to lamin C ratio determines the stiffness of the nucleus and, thus, resistance to deformation. **c**, Nuclei of fibroblasts exposed to soft environments exhibit densely packed chromatin, high levels of repressive histone methylation, low levels of activating histone methylation, low histone acetylation and low DNA methylation. The implications of mechanically regulated epigenetic modifications are just beginning to be understood. **d**, Conversely, nuclei of myofibroblasts in stiff environments display a less compact chromatin structure, with low levels of repressive histone methylation but high activating histone methylation, histone acetylation, and DNA methylation levels. **e**, Epigenetic modification of the promoter regions of pro-fibrotic and anti-fibrotic genes by various epigenetics-modifying enzymes drive persistent myofibroblast activation and fibrosis. Enzymes, such as ASH1, the methyltransferase (MET) COMPASS complex, mixed-lineage leukaemia protein 1 (MLL1) and p300, place transcription-activating histone methylation and acetylation marks that promote the expression of pro-fibrotic genes. Conversely, anti-fibrotic promoter regions are characterized by the removal of active histone acetylation marks by histone deacetylases (HDACs; for example, HDAC8) and the addition of repressive histone methylation marks by enzymes such as EZH2 and EHMT2. DNA methylation (5mC) by DNA methyltransferases (DNMTs) is associated with suppression of the expression of both pro-fibrotic genes (*Acta2*, *Col1a1*, *Col2a1*, *Tgfb1*, *Timp1*) and anti-fibrotic genes (*Pparg*, *Pgc1a*, *FLIT*, *Socs3*, *Dkk1*, *Sfrp1*, *Thy1*, *Ptger2*, *Cox2*, *P14arf*). CBX5, chromobox protein homologue 5.

and cytoskeleton (LINC) complex in the outer nuclear membrane. For recent reviews on nuclear structures and nuclear mechanotransduction, see refs. 243–245. The LINC complex spans the nuclear envelope and is composed of nesprins and Sad1 and UNC-84 (SUN) proteins. The LINC complex binds to lamins in the inner nuclear membrane, which directly interact with chromatin. Interaction of LINC with actin and vimentin filaments establishes a mechanical axis that transmits mechanical information from the cell surface to the nucleus. The presence of such an axis has been elegantly shown by pulling DNA out of the nucleus using micromanipulated microbeads that engaged cell surface integrins[246]. Mechanical deformation of the nucleus alters chromatin organization initially by softening the nuclear envelope to prevent DNA damage[247,248]. This is followed by conversion of DNA from a condensed to an opened state, which also allows access for gene transcription or DNA repair factors[249]. For instance, nuclear stress affects the transcriptional regulation of fibrotic genes of mesenchymal cells on stiff substrates and promotes myofibroblast activation in different cells and organs, which is in part due to the state of chromatin condensation, in addition to control by signalling as described in Fig. 2 (refs. 237,239). Conversely, some of these studies have shown that disrupting the LINC complex in MSCs grown on stiff substrates results in high DNA condensation and prevents myofibroblast activation[238]. Because local stretching of the nucleus of Chinese hamster ovary lineage cells rapidly enhances the expression of genes in the correspondingly strained DNA region[249], it is tempting to speculate that similar mechanisms apply to the regulation of mechanically responsive myofibroblast genes.

Epigenetic control of myofibroblast persistence

Compared to mechanical stress, histone acetylation is a more widely studied epigenetic mechanism controlling chromatin density, that is, accessibility of DNA regions for the binding of transcription factors. Although histone acetylation marks are typically associated with active gene transcription[225], their role in controlling myofibroblast persistence is versatile. Reducing histone acetylation by inhibiting p300 (ref. 250) or increasing histone acetylation using inhibitors of HDACs[251] both attenuate the expression of myofibroblast markers and fibrosis in animal models of cardiac injury. Consistently, skin biopsies and explanted dermal fibroblasts from patients with scleroderma show higher levels of the HAT p300 compared to fibroblasts from healthy individuals[252]. The presence and absence of histone acetylation marks can change the expression of fibrosis effector genes directly or indirectly by modulating the expression of regulators of fibrosis (Fig. 3). One example of direct regulation is the p300-mediated hyperacetylation of the histone H3 (H3K27), which can be pharmacologically inhibited to reduce myofibroblast activation (expression of *ACTA2*, *COL1A1*, *COL15A1*, *POSTN*) of fibroblasts isolated from the fibrotic palm tissue of patients with Dupuytren disease[253]. Likewise, p300 HAT levels are elevated in the skin of patients with systemic sclerosis; experimentally enhanced levels of p300 HAT are associated with hyperacetylation of histone H4 at promoter regions of *COL1A2* (encoding collagen type I, α2) in scleroderma fibroblasts, which could be blocked using selective inhibitors of p300 HAT[252]. The same study showed that TGFβ is a key inducer of *EP300* (encoding p300) expression by enhancing binding of the transcription factor early growth response protein 1 (Egr1) to the *EP300* promoter[252]. Conversely, H3K27 de-acetylation at promoter regions of anti-fibrotic genes, such as *PPARG* (encoding peroxisome proliferator-activated receptor-γ)[254] by HDAC8 and *PGC1A* (encoding peroxisome proliferator-activated receptor-γ co-activator 1α)[255] by HDAC7, leads to their reduced expression in TGFβ-activated lung fibroblasts. Indirect regulation of myofibroblast activation by histone acetylation marks was described for HDAC4, which mediates the elevated expression of *ACTA2* (encoding αSMA) in TGFβ-treated cultured lung fibroblasts[256]. General inhibition of HDACs was shown to inhibit myofibroblast activation through reduced phosphorylation and nuclear translocation of signal transducer and activator of transcription 3 (STAT3) in renal interstitial fibroblasts in vivo[257]. It seems that cytoplasmic–nuclear translocation of HDAC4 during myofibroblast activation is also driven by mechanical stress: mouse embryonic fibroblasts grown on stiff substrates (100 kPa) showed higher nuclear levels of HDAC4 and higher *HDAC4* expression than fibroblasts grown on soft substrates (0.5 kPa)[228]. Analogously, stiff (25.6 kPa) but not soft

culture environments (0.4 kPa) promoted the phosphorylation and subsequent nuclear translocation of the HAT p300 through enhanced AKT-dependent signalling during myofibroblast activation of human hepatic stellate cells (HSCs)[258].

Histone methylations can either enhance or silence gene transcription, depending on the location of the methyl mark in specific lysine residues of the histone tail. In general, histone 3 lysine 9 trimethylation (H3K9me3) and H3K27me3 are repressive methylation marks, whereas H3K4me3, H3K36me2 or H3K3me3, and H3K79me3 activate gene transcription[226]. One of the few examples of inhibition of myofibroblast activation by repressive histone methylation of pro-fibrotic genes is the silencing of the transcription factor Fos-related antigen 2 (Fra2) by H3K27me3 (ref. 259). However, in most cases, myofibroblast activation is achieved by activating histone methylation marks in pro-fibrotic and ECM-related genes[260–262]. Consequently, activated myofibroblasts isolated from fibrotic rat livers exhibit higher global levels of active histone methylation marks (H3K4me2 and/or H3K4me3) and lower levels of repressive histone marks (H3K9me3 and H3K27me2) compared to quiescent HSCs from healthy liver[260]. These methylation changes are accompanied by enhanced expression of three H3K4-specific METs: mixed-lineage leukaemia protein 5 (MLL5; also known as KMT2E), histone-lysine N-methyltransferase SETD1A and ASH1 (ref. 260). ASH1 is recruited to the proximal promoters of *Acta2*, *Timp1* (encoding metalloproteinase inhibitor 1), *Col1a1*, and *Tgfb1* and enhances their expression by conferring the active histone mark H3K4me3 (ref. 260). Activating H3K4me3 or H3K4me2 marks in the promoter regions of *Col1a1*, *Col1a2* and *Acta2* are further enriched by the MET complex COMPASS in TGFβ-activated HSCs[261]. Likewise, enrichment of MLL1 binding to the promoters of ECM-related genes results in H3K4 trimethylation and drives HSC–myofibroblast activation in experimental liver fibrosis[262].

In some cases, myofibroblast activation is also achieved by repressive histone methylation of the promoters or exons of genes that encode anti-fibrotic factors such as PPARγ (Fig. 3e). For instance, the MET EZH2 methylates H3K27 and thereby forms a repressive structure in regions downstream of the *Pparg* exon as shown in experimental models of induced liver fibrosis[263]. Likewise, experimentally knocking down the histone-lysine demethylase 3A (KDM3A; also known as JMJD1A or JHDM2A) allowed repressive H3K9me2 to accumulate in the *Pparg* gene promoter, thereby enhancing myofibroblast activation of HSCs in culture and in a rat model of induced liver fibrosis[264]. Myofibroblast activation of rat renal fibroblasts is promoted by repressive H3K9 methylation of regulatory gene regions of the TGFβ inhibitor Klotho by the histone-lysine N-methyltransferase EHMT2 (also known as MET G9). Consistently, high levels of EHMT2 and H3K9me1 and low levels of Klotho have been measured in kidney biopsies of patients with tubulointerstitial fibrosis[265]. EHMT2 is also involved in lung myofibroblast activation by forming a complex with the epigenetic repressor chromobox protein homologue 5 (CBX5) to repress the promoter of the anti-fibrotic gene *PGC1a* in H3K9me[236]. The expression of EHMT2 itself is regulated through TGFβ1–SMAD3 signalling in renal fibroblasts[265], similar to TGFβ1-induced expression of the METs SET7 and SET9 in myofibroblast-activated mesangial cells[265].

There are some indications for mechanical control of MET activities through MRTFA: the presence of MRTFA binding sites in the promoters of some pro-fibrotic genes, such as *ACTA2*, led to the discovery that MRTFA recruits METs (for example, COMPASS) in TGFβ-treated HSCs and, consequently, that depletion of MRTFA led to reduced H3K4 methylation and reduced myofibroblast activation[261]. Further complexity is added by the functions of histone methylations that are beyond conventional gene regulation. For instance, global H3K9me3-marked heterochromatin was recently shown to protect the DNA of epithelial cells from mechanically induced damage[248]. It is tempting to speculate that myofibroblasts might use epigenetic modifications to globally protect their genome from the high stresses and strains arising during tissue remodelling but this is yet to be confirmed.

Control of myofibroblast persistence by DNA methylation

In addition to histone modifications, DNA methylation is one of the most powerful epigenetic drivers of organ fibrosis, where persistent myofibroblasts often exhibit global DNA hypermethylation[266,267]. DNA methylation is performed by DNA methyltransferases (DNMTs), which add methyl groups to cytosines in CpG islands (Fig. 3c–e); thus, DNA methylation in myofibroblasts is controlled through regulation of DNMT expression levels. DNMT1 and DNMT3a are upregulated in scleroderma skin and induced by pro-fibrotic TGFβ in dermal fibroblasts[268]. In a process mediated by hypoxia-inducible factor 1α (HIF1α), DNMT1 and DNMT3b are upregulated during cardiac fibrosis, which can be mitigated by knockdown of DNMT3b[269]. DNMT1 is also highly expressed in lung and kidney fibrosis, where it is a hallmark of myofibroblast persistence[270,271]. The DNMT inhibitor 5-azacytidine reduces fibrosis in various animal models[268,269,272]. Because DNA methylation generally promotes gene silencing by creating binding sites for repressor proteins in gene promoters[225], myofibroblast activation from mesenchymal precursor cells is typically achieved by hypermethylation and thus by silencing of fibrosis-suppressing genes. Examples of DNA methylation-regulated anti-fibrotic genes are *FLI1*, *Socs3*, *Dkk1*, *SFRP1* (ref. 273), *Thy1* (ref. 274), *PTGER2* (encoding prostaglandin E receptor 2 (ref. 275)) and *Ptgs2* (encoding cyclooxygenase 2)[276]. Hypermethylation can further render myofibroblasts resistant to apoptosis through the silencing of *P14ARF*, which arrests the cell cycle and promotes myofibroblast persistence in fibrotic lungs[277]. One exciting option to therapeutically control myofibroblast fate related to DNA methylation is the use of inhibitors directed against specific epigenetic regulators, for instance, members of the methyl-CpG-binding domain (MBD) family and the bromodomain and extra-terminal domain (BET) families of epigenetic readers[263,278] (Supplementary Box S4).

In addition to the hypermethylation of myofibroblast-suppressing genes, myofibroblast-associated genes have been reported to be hypomethylated in fibrotic conditions, most notably for *Acta2*. Reducing DNMT activity and thereby DNA methylation of the *Acta2* promoter was shown to result in lung fibroblast activation[279]. However, rather than serving as a mechanism regulating fibroblast–myofibroblast activation, *Acta2* hypomethylation is thought to prevent αSMA expression in non-mesenchymal cells, such as alveolar epithelial type II cells in the lungs[279]. Nevertheless, DNA hypomethylation was reported to support the acquisition of mesenchymal features in cultured cardiac endothelial cells after treatment with TGFβ1 and in experimental heart fibrosis[271,280].

In contrast to the emerging connection between nuclear mechanics, chromatin condensation and histone modifications, it is still unclear whether and how mechanical stress can regulate DNA methylation. In liver fibrosis, expression of the nuclear membrane component SUN2 is downregulated by DNMT3b-mediated hypermethylation with adverse effects on genome stability, leading to pro-fibrotic effects[281]. ECM stiffness was shown to co-regulate DNMT1 activity together with myofibroblast-related phenotypes of SMCs[282]. Furthermore, the *DNMT1* promoter contains binding motifs for MRTFA[283],

and YAP1 was shown to regulate DNMT1 expression in liver cancer cells[284], all suggesting potential regulation of DNA methylation by mechanical factors.

Conclusions and perspectives

Here, we reviewed the biological mechanisms of myofibroblast activation focusing on the impact of the mechanical environment and signal mechanotransduction. The involved mechanisms and factors present potential targets for therapeutic intervention to block myofibroblast formation and/or persistence. Indeed, some of these conceptual targets have progressed into clinical trials such as ECM-crosslinking enzymes, contraction mediators and specific integrins. We refer to recent reviews that provide clinically inclined scientists — or patients, for that matter — with the translational aspects of anti-myofibroblast and anti-fibrotic strategies[13,14,285,286].

Although central to the physiological and pathological tissue repair process, myofibroblasts do not act in isolation but in concert with multiple other cell types. The role of fibroblast and myofibroblast communication with innate and adaptive immune cells, epithelium cells, and vascular cells in driving tissue repair and fibrosis has been reviewed elsewhere[119,285,287]. Accumulating evidence shows that normal and fibrotic repair engage very similar if not identical cell activation states. However, whether scar tissue accumulates or not depends on the spatial and temporal circumstances under which these activation states occur[288,289]. One example of the importance of spatiotemporal coordination in wound environments is the crosstalk between fibroblastic cells and macrophages, which has (re-)gained increasing interest in the past years. Macrophage–myofibroblast crosstalk has been described for various fibrotic organs and is suggested to promote fibrosis by locally regulating the activation state of fibroblastic cells[290–295].

Macrophages, just like fibroblastic cells, acquire different functional 'polarization' states over the course of tissue repair[295]. Macrophages with both pro-inflammatory or pro-fibrotic and pro-repair functions are crucial for the successful repair and scar resolution of injured tissues (Fig. 1a). An imbalance in these functions or mistiming of the associated macrophage polarization states can result in either non-healing wounds[296,297] or fibrosis[295,298,299]. This is in part due to the impact that different macrophages have on the activation state of fibroblastic cells. For instance, experimental depletion of macrophages in healing mouse skin wounds stimulates the expansion of a myofibroblast subpopulation that is associated with dermal repair[300]. Conventionally, macrophages are known to control fibroblast survival, proliferation, collagen production and myofibroblast activation through the paracrine secretion of cytokines[85,294,295,301–304]. More recently, direct contact has been shown to be critical for specific molecular signals to pass between macrophages and myofibroblasts. Our own studies support the notion that physical contact between fibroblasts and pro-fibrotic macrophages is maintained by heterocellular junctions and creates a niche of active TGFβ1 that induces and maintains myofibroblasts in their highly contractile, αSMA-positive state[305]. We are only beginning to understand the molecular interactions at play in such heterocellular niches, the study of which is an emerging field further driven by the power of spatial transcriptomics[41]. It is already clear that it will unlikely suffice to target single cell types in anti-fibrosis therapies without considering the spatiotemporal context of the condition.

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